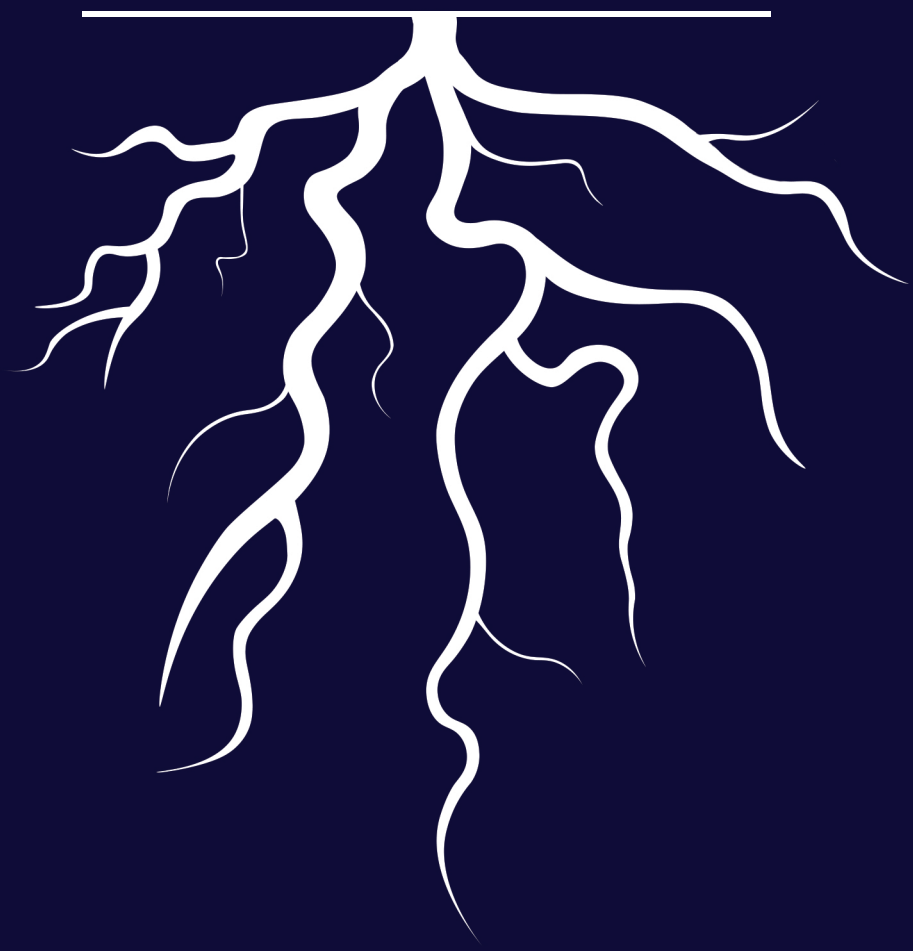
TARGETING ADRENOMEDULLIN IN SEPSIS



Christopher Geven

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Colofon

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Promotor
Prof. dr. Peter Pickkers

Copromotor
Dr. Matthijs Kox

Manuscriptcommissie
Prof. dr. Niels Riksen
Dr. Quirijn de Mast
Prof. dr. Mervyn Singer (University College London, Verenigd Koninkrijk)

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Christopher Bernardus Carolus Alexander Gerardus Geven

born on September 29, 1990
in Asten (the Netherlands)

Supervisor
Prof. dr. Peter Pickkers

Co-supervisor
Dr. Matthijs Kox

Doctoral thesis committee
Prof. dr. Niels Riksen
Dr. Quirijn de Mast
Prof. dr. Mervyn Singer (University College London, United Kingdom)

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Chapter 1

General introduction and outline of this thesis

A brief history of sepsis

Since ancient times, humanity has been plagued by pathogenic microorganisms. Infectious disease has been a leading cause of death that contributed significantly to a shorter average lifespan due to high infant mortality and many population-decimating plagues (e.g. the bubonic plague 'Black Death' in the 14th century, estimated to kill 30-60% of Europe's total population). The term 'sepsis' originates from the Greek word 'σηψις' (rotting, putrefying or decaying)[1], and was first encountered in Homer's Iliad (c. 750 BC)[2]. It was also found in the medical writings of the Greek physician Hippocrates (c. 460-377 BC).

The oldest known report on sepsis dates back to an Egyptian papyrus manuscript written around 1600 BC (and appears to be a copy of a manuscript from 3000 BC)[3]. It summarizes 48 cases of traumatic lesions and wounds, explaining their signs and symptoms, treatment and follow-up (**Figure 1**). In a few of these cases, fever and pus are clearly described as secondary phenomena and associated with an unfavourable prognosis.

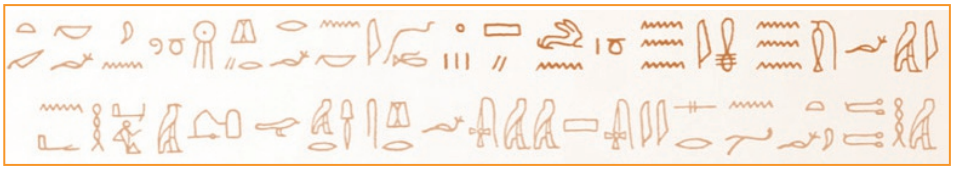

Figure 1. Hieroglyphs (lines 10-11, case 47) from the Edwin Smith surgical papyrus (bought by Edwin Smith in 1862 in Luxor, Egypt). Described is the disease-course of a patient with a shoulder wound. On the third examination of the patient, the physician observed an inflamed wound and fever (which a modern physician would likely diagnose as an infection). Next, he declared the patient's condition critical. Translation of depicted hieroglyphs: *'Thou shouldst say concerning him: One having a gaping wound in his shoulder, it being inflamed, and he continues to have fever from it. An ailment with which I will contend.'* Adapted from: J.H. Breasted. The Edwin Smith Surgical Papyrus. 1980. Chicago University, Chicago Press.

One of the earliest thoughts of disease - the concept of dysregulated bodily humors as a cause of disease - was put forth by Hippocrates[4]. In addition, the early Greeks and Romans believed that bad or corrupt air (later termed 'miasma') could cause pestilence, and physician Galenus traced individual susceptibility to miasma to the balance of humors in the body. These early concepts remained largely unchallenged for over two millennia.

The existence of microorganisms as a cause of disease was hypothesized by scholars such as Marcus Terentius Varro[4] (c. 100 BC), long before their actual discovery. It was not until the 17th century that scientists such as Antonie van Leeuwenhoek and Robert Hooke were the first to actually observe microbial life through their microscopes, paving the way for major scientific discoveries such as the germ-theory of disease (Louis Pasteur and Robert Koch), desinfection[5,6], vaccination[7], anti-septic surgical procedures[8] and antimicrobial therapies[9-11].

In the 20th century, researchers began unravelling the pathogenesis of sepsis. Several remarkable discoveries have increased our understanding of the pathophysiology of sepsis, such as the existence of (endo)toxins[12], cytokines[13] and pattern recognition receptors[14], the importance of the coagulation system[15] and endothelium[16], the role of substances such as nitric oxide[17] and catecholamines[18], as well as technical advances, for example in hemodynamic monitoring[19-21].

In order to promote early sepsis detection in patients, the SIRS (Systemic Inflammatory Response Syndrome) criteria were adopted by the medical community in 1992[22]. SIRS was defined as a pro-inflammatory state that affected the whole body and could be evoked by an infectious or non-infectious insult. Sepsis was present when two or more SIRS criteria were observed together with a suspected or documented infection. However, later studies showed that the SIRS criteria have important limitations, including poor specificity (i.e. many patients were falsely diagnosed with sepsis) and sensitivity (i.e. sepsis can be present in the absence of SIRS criteria)[23]. Given the emphasis on 'pro-inflammation' at that time, the general accepted idea was that patients died as 'collateral damage' of their own overzealous immune response. Many studies aimed to improve sepsis outcomes by inhibiting pro-inflammation through pharmacological interventions (e.g. TNF-α and LPS antibodies, IL-1 inhibitors, etc.), often targeting only a single molecule or pathway. Overall, in non-selected sepsis patients, none of these interventions were proven to be effective, indicating that the disease is more complex than is often assumed, with a large patient heterogeneity and many pathways and mediators leading to injury. Recently, new sepsis definitions were put forth that recognize the limitations of SIRS and shift the focus from pro-inflammation to organ dysfunction in patients with a dysregulated immune response[24]. In addition, sepsis-induced immunoparalysis - a hypoinflammatory state

characterized by profound immunosuppression - is increasingly recognized to play a role in the later phase of sepsis, making patients more vulnerable to opportunistic secondary infections[25,26].

Sepsis as we know it today

In present times, sepsis remains a major worldwide health issue with an estimated 30 million episodes and 6 million deaths per year, although this number is probably a significant underestimation[27,28]. The World Health Organization recently made sepsis a global health priority by adopting a resolution to improve, prevent, diagnose and manage sepsis. In the latest definitions, sepsis is characterized as a systemic, dysregulated host response to infection that leads to life-threatening organ dysfunction[24]. Septic shock is considered a subset of sepsis in which extensive circulatory, cellular, and metabolic abnormalities are associated with an even greater mortality risk. In the clinical setting, septic shock is characterized by elevated plasma lactate concentrations and the need for vasopressors to maintain sufficient blood pressure, despite adequate fluid resuscitation.

Due to the nature and inherent risks of sepsis and septic shock, the syndrome should be considered a medical emergency and treatment should be started as soon as possible[29]. Cornerstones of sepsis treatment are source control, antibiotic therapy, hemodynamic stabilisation through fluid resuscitation and vasopressor therapy, as well as organ support (e.g. mechanical ventilation and renal replacement therapy).

Despite advances in the knowledge of sepsis pathophysiology and refinement of existing therapies, the mortality of sepsis remains very high as one out of every three to four patients die[30]. As mentioned before, no new sepsis-specific adjuvant therapies were proven to be effective. Therefore, exploration of other treatment targets for sepsis is highly warranted.

The vascular endothelium might be an attractive target for pharmacological interventions[31]. The vascular endothelium consists of a single layer of cells that lines the interior of the blood vessel. Its main function is to control diffusion of molecules and cells between the intravascular and interstitial space. Endothelial dysfunction is one of the primordial hallmarks of sepsis[16]. During sepsis, the endothelium may become compromised due to the profound inflammatory response, resulting in endothelial cell death and loss

of barrier integrity[32,33]. This can result in extravascular accumulation of fluids and molecules, causing edema, hemodynamic instability and subsequent organ failure.

Adrenomedullin, a key vasoactive hormone in sepsis

Adrenomedullin was discovered by Japanese researchers (Kitamura and colleagues) in 1993 who were searching for vasoactive peptides in tissue extracts originating from human pheochromocytoma[34]. The researchers identified a novel 52-amino acid peptide that induced a profound cyclic adenosine monophosphate (cAMP) response in platelets and subsequently named this peptide hormone 'adrenomedullin', because the pheochromocytoma originated from the adrenal medulla. Interestingly, adrenomedullin is ubiquitously expressed in animals belonging to the biological kingdom 'animalia'. So far, over 193 species belonging to the subphylum 'vertebrata' were found to have orthologs to adrenomedullin (i.e. genes in different species that evolved from a common ancestral gene by speciation, generally retaining the same function in the course of evolution), including amphibians, birds, cetacea and mammals. Moreover, highly comparable amino acid sequences of adrenomedullin are found across different species (**Figure 2**). The fact that the gene and amino acid sequence have been retained throughout evolution in highly different species suggests that adrenomedullin is an important, perhaps essential peptide.

Figure 2. Adrenomedullin amino acid sequence in humans[35], Cynomolgus monkeys[36], dogs[37], mice[38] and chickens[38]. Underscored letters indicate a different amino acid sequence compared to humans.

Preclinical studies have confirmed the importance of adrenomedullin, as deletion of crucial parts of the adrenomedullin signalling pathway in knockout models were shown to result in lethal hydrops fetalis, indicating inadequate development of the vascular endothelial barrier[39,40]. Adrenomedullin levels are increased in sepsis patients and correlate with disease severity and mortality[41,42]. However, it is of importance to emphasize that correlation does not imply causation, and therefore these observational studies do not reveal whether the increased concentrations of adrenomedullin may be beneficial or detrimental in sepsis patients. Mechanistic *in vitro* and *in vivo* studies have

shown that adrenomedullin exerts many different effects in different organ systems and cells, including vasorelaxation of vascular smooth muscle cells and stabilization of the endothelial barrier. Preclinical work in animal models of systemic inflammation and septic shock showed that administration of adrenomedullin is able to beneficially influence outcome. However, given its strong vasodilatory effects (thereby potentially augmenting shock) and short half-life, adrenomedullin infusion may not be ideal for use in patients.

Adrenomedullin as a treatment target

Previously, various high-affinity monoclonal antibodies were developed, each targeting different epitopes of the adrenomedullin peptide, resulting in complete or partial inhibition of adrenomedullin signaling[43]. The efficacy of these antibodies was investigated in a lethal model of murine septic shock[43]. Interestingly, a non-neutralizing antibody directed against the N-terminal epitope of adrenomedullin resulted in a survival benefit, whereas antibodies targeting the C-terminal epitope that completely inhibited adrenomedullin's function did not. Subsequent studies with this non-neutralizing antibody in a resuscitated model of murine septic shock showed that pretreatment with the antibody improved various outcome parameters, including reduced catecholamine demand and renal dysfunction[44].

Subsequently, a humanized variant of this murine antibody has been developed, and was named Adrecizumab. The name Adrecizumab can be broken down into *Adre-ci-zu-mab*, marking it as a humanized ('zu') monoclonal antibody ('mab') acting on the cardiovascular system ('ci'), targeting adrenomedullin ('Adre'). The preclinical and first-in-human safety, efficacy and pharmacokinetics/-dynamics of this novel, humanized, adrenomedullin-binding antibody are presented in this thesis.

Aims of this thesis

- To provide an overview of adrenomedullin's effects in the context of sepsis and heart failure
- To unravel the mechanism of action of Adrecizumab
- To provide a detailed overview of experimental human endotoxemia
- To assess the association of adrenomedullin levels and outcome in critically ill and sepsis patients
- To evaluate the safety and efficacy of Adrecizumab in both animals and humans

Outline of this thesis

Part I provides in-depth background information on adrenomedullin and the adrenomedullin-binding antibody Adrecizumab in the context of sepsis and heart failure, as well as background information on the experimental human endotoxemia model. **Chapter 2** provides information on endothelial dysfunction and the role of adrenomedullin during sepsis. Furthermore, the use of adrenomedullin as a therapy or treatment target is discussed. In **chapter 3** and **chapter 4** a hypothesis on the mechanism of action of Adrecizumab is presented, which may have therapeutic potential in sepsis patients. **Chapter 5** describes the potential role of adrenomedullin in heart failure, together with a hypothesis on the potential therapeutic use of Adrecizumab in heart failure patients. Part I ends with **chapter 6**, in which an extensive description of experimental human endotoxemia is provided, a model of systemic inflammation in humans that is also used in part IV of this thesis.

Recently, a novel assay has been developed to measure circulating levels of biologically active adrenomedullin (bio-ADM)[45]. Two initial studies revealed increased bio-ADM concentrations in sepsis, which correlated with disease severity and mortality[41,42]. **Part II** contains two chapters on the use of bio-ADM as a biomarker in a general critically ill population (**chapter 7**) and in patients with severe sepsis and septic shock (**chapter 8**).

Part III focuses on preclinical evaluation of Adrecizumab. In **chapter 9**, the effects of Adrecizumab on vascular barrier function and survival in rodent models of systemic inflammation and sepsis are described. In **chapter 10**, the effects of Adrecizumab on hemodynamics and the myocardium are investigated in septic rats. **Chapter 11** contains a preclinical safety evaluation of Adrecizumab in rodents, beagle dogs and non-human primates.

Part IV describes the first-in-human clinical evaluation of Adrecizumab. Two phase I studies in healthy human volunteers are described in **chapter 12**. First, the safety, tolerability and pharmacokinetics/-dynamics of Adrecizumab were investigated during non-inflammatory conditions. Subsequently, a study with Adrecizumab was performed during systemic inflammation induced by experimental human endotoxemia. **Chapter 13** comprises the study protocol of the currently ongoing phase II, randomized, double-blind, placebo-controlled study with Adrecizumab in patients with early septic shock (AdrenOSS-2).

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Part I

Background

Chapter 2

Adrenomedullin and adrenomedullin-targeted therapy as treatment strategies relevant for sepsis

Christopher Geven, Matthijs Kox and Peter Pickkers

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Abstract

Sepsis remains a major medical challenge, for which, apart from improvements in supportive care, treatment has not relevantly changed over the last few decades. Vasodilation and vascular leakage play a pivotal role in the development of septic shock, with vascular leakage being caused by disrupted endothelial integrity. Adrenomedullin (ADM), a free circulating peptide involved in regulation of endothelial barrier function and vascular tone, is implicated in the pathophysiology of sepsis. ADM levels are increased during sepsis, and correlate with extent of vasodilation, as well as with disease severity and mortality. In vitro and preclinical in vivo data show that administration of ADM exerts anti-inflammatory, antimicrobial, and protective effects on endothelial barrier function during sepsis, but other work suggests that it may also decrease blood pressure, which could be detrimental for patients with septic shock. Work has been carried out to negate ADM's putative negative effects, while preserving or even potentiating its beneficial actions. Preclinical studies have demonstrated that the use of antibodies that bind to the N-terminus of ADM results in an overall increase of circulating ADM levels and improves sepsis outcome. Similar beneficial effects were obtained using coadministration of ADM and ADM-binding protein-1. It is hypothesized that the mechanism behind the beneficial effects of ADM binding involves prolongation of its half-life and a shift of ADM from the interstitium to the circulation. This in turn results in increased ADM activity in the blood compartment, where it exerts beneficial endothelial barrier-stabilizing effects, whereas its detrimental vasodilatory effects in the interstitium are reduced. Up till now, in vivo data on ADM-targeted treatments in humans are lacking; however, the first study in septic patients with an N-terminus antibody (Adrecizumab) is currently being conducted.

Introduction

Sepsis remains a major health problem in the 21st century, with an increasing incidence and high mortality in intensive care units worldwide[1,2]. Sepsis is an inflammatory syndrome in which a dysregulated host response to infection results in life-threatening organ dysfunction[3]. Its most severe form, septic shock, is defined by increased lactate levels and vasopressor requirement to maintain sufficient blood pressure and organ perfusion, despite adequate fluid resuscitation[3]. The sepsis syndrome is characterized by a very complex, multilayered pathogenesis, that involves many harmful and protective pathways[4,5]. The vascular endothelium is a protective barrier involved in the maintenance of vessel integrity that controls diffusion of molecules between the intravascular and interstitial space. Endothelial dysfunction is one of the major hallmarks of sepsis[6]. The profound inflammatory response observed in sepsis plays a pivotal role in this phenomenon, which is accompanied by endothelial cell death and loss of barrier integrity[5-8]. Underlying processes of loss of barrier integrity include increased actomyosin contraction (also known as 'stress fiber formation') in response to phosphorylation of myosin light chains by myosin light chain kinase (MLCK)[9]. Loss of barrier integrity leads to extravascular accumulation of fluids and molecules, causing edema, a decreased blood pressure and subsequent organ failure. A considerable percentage of mortality occurs in the early phase of sepsis, when multi-organ failure develops despite supportive therapies. Although the general knowledge of the pathophysiology of sepsis has improved, this has not translated to a single effective adjuvant therapy. The lack of clinical trials that show a therapeutic benefit may partially be explained by large patient heterogeneity, but also because of the complexity of the pathophysiology[8,10]. Thus, there is still an urgent and unmet need for new therapeutic options, and interventions that may improve the endothelial barrier function and vascular tone are an attractive category[11]. A key hormone involved in regulation of the endothelium barrier and vascular tone is adrenomedullin (ADM). In the current review, we describe the general vascular properties of ADM and provide an overview of the current understanding of the role of ADM in sepsis and septic shock. Furthermore, we discuss the potential of ADM and ADM-targeted treatments for sepsis patients.

Adrenomedullin

ADM was first discovered in human pheochromocytoma tissue in 1993[12]. Although ADM's initially discovered effects were vasodilation and blood pressure lowering effects[12-14], later work demonstrated that ADM exerts a multitude of biological actions, in both health and disease[15,16]. ADM is a 52 amino acid peptide belonging to the calcitonin gene-related peptide family[17]. In humans, the gene encoding ADM is located on chromosome 11 and consists of 4 exons and 3 introns[18]. The gene is transcribed into a pre-messenger RNA (mRNA) molecule, containing 4 exons and 3 introns. The removal of all introns results in the formation of a mature mRNA molecule (form A) which is eventually translated and processed into ADM as detailed below. However, if the third intron of this pre-mRNA molecule is not removed, this results in the formation of a longer mRNA molecule (form B). Due to the presence of a stop codon in this intron, a smaller prohormone is produced that does not result in the production of ADM[19]. It remains unknown which factors regulate the splicing of this third intron and whether this is altered during sepsis. Translation of the form A mRNA molecule leads to a 185 amino acid long preprohormone (prepro-ADM) that undergoes a multistep cleavage process. First, a 21-residue N-terminal signalling peptide is cleaved of prepro-ADM, generating a 164 amino acid pro-ADM peptide. Next, pro-ADM is cleaved into pro-ADM N-terminal 20 peptide (PAMP)[20-22], midregional pro-ADM (MR-proADM)[23], adrenotensin[24] and a glycine-extended 53-amino acid peptide, the latter of which is subsequently converted to the 52-amino acid mature ADM by enzymatic amidation to an extent, which may vary depending on the pathology and other factors[25]. Besides ADM, several of the other cleavage products are also vasoactive (e.g. PAMP exerts vasodilatory effects, whereas adrenotensin is vasoconstrictive). ADM is widely expressed in virtually all human tissues. The highest concentrations of the peptide were found in the adrenal medullae, cardiac atria and lungs[26,27], whereas the highest concentrations of ADM mRNA were measured in the lungs, cardiac atria, aorta and mesenteric arteries[28]. Many cells are capable of producing ADM, including endothelial cells, vascular smooth muscle cells (VSMCs), monocytes, renal parenchymal cells and macrophages[29-35]. ADM exerts its effects by ligation of receptor complexes consisting of the calcitonin receptor-like receptor (CRLR) combined with a specific receptor activity-modifying protein (RAMP)[36,37]. The ADM1 and ADM2 receptors consist of the CRLR combined with RAMP2 and RAMP3, respectively, whereas the combination of CRLR and RAMP1 forms the CGRP (calcitonin gene-related protein)

receptor. Most functional studies do not specify which receptor is specifically activated, and are therefore referred to as 'ADM receptors'. Analogous to the ubiquitous expression of the ADM peptide, ADM receptors have also been detected in various tissues and organs, including blood vessels, skeletal muscles, heart, lungs and nerve tissue[38-41]. On a cellular level, ADM receptors are expressed on many different cell types, including endothelial cells, vascular smooth muscle cells, cardiomyocytes, macrophages and dendritic cells[33,42-44]. Interaction of ADM with its receptor occurs through its C-terminal moiety[45], and the N-terminal part of ADM is thought to be only of minor importance for its agonist function[46]. Circulating ADM has a half-life of approximately 22 minutes[47], and is rapidly degraded from its N-terminus by proteases[48-50]. Moreover, it has been reported that the three CRLR/RAMP receptors are internalized upon stimulation together with ADM, and thus function as clearance receptors[51,52]. On a more organ specific level, the lungs appear to be involved as a site of clearance[53,54].

The role of adrenomedullin in the regulation of blood pressure

As mentioned before, the first discovered physiological effect of ADM was vasodilation, leading to hypotension and reduced peripheral resistance[12-14]. Over the following years, many studies have confirmed these results. *In vitro* studies demonstrated potent vasodilatory effects of ADM on isolated blood vessels[42,55] and in isolated organs[56], and *in vivo* studies showed that direct infusion of ADM resulted in decreased blood pressure and induced a compensatory increase of heart rate, endogenous noradrenaline and renin concentrations in various mammalian species (including humans), which coincided with increased cardiac output[14,57-62]. These vasodilatory effects of ADM are mediated through binding with its receptors present on vascular endothelial cells and VSMCs[42]. It is unknown, though, how both types of interaction contribute quantitatively under physiological and pathophysiological conditions to vasodilation. Several signaling pathways have been described through which ADM causes vasodilation, both endothelium-dependent and endothelium-independent[55], which are depicted in **Figure 1**. In an endothelium-independent way, binding of ADM with its receptors on VSMCs increases intracellular cyclic adenosine monophosphate (cAMP)[63,64], which subsequently activates protein kinase A (PKA, also known as cAMP-dependent kinase)[42]. PKA inhibits smooth muscle cell contraction in several ways. For example, it induces the opening of vascular potassium channels, causing potassium efflux, leading to subsequent membrane potential

hyperpolarisation and closing of voltage gated calcium channels, ultimately reducing intracellular calcium content[42,65-67]. Of note, potassium channel activation is known to play an important role in the blunted norepinephrine-responsiveness observed in sepsis[68], and potassium channel blockers have been shown to restore norepinephrine sensitivity in a human *in vivo* model of systemic inflammation[69]. Other effects of PKA include inhibition of sarcoplasmatic calcium channels and MLCK. The endothelium-dependent mechanisms through which ADM induces vasodilation are the inositol-1,4,5-triphosphate (IP_3) system and the phosphatidylinositol-4,5-bisphosphate 3-kinase-protein kinase B (PI3K/Akt) pathways. Both of these pathways stimulate endothelial nitric oxide (NO) synthase (eNOS), leading to NO release. In turn, NO activates cyclic guanoside monophosphate (cGMP) in VSMCs, resulting in activation of protein kinase G (PKG), ultimately leading in inhibition of sarcoplasmatic calcium channels and activation of myosin light chain phosphatase[69,70] and vasodilation. Prostaglandins have also been linked to ADM-induced vasodilation, through the endothelium-dependent cyclooxygenase-1 (COX-1) pathway[42,71], although results are inconsistent[66], which may be due to differences between animals and the origin of the vessels studied. Finally, it has been suggested that ADM is involved in the central regulation of blood pressure, although these data are equivocal. The presence of ADM has been demonstrated in the hypothalamus[72], and some studies have reported that micro-injections of ADM into the hypothalamic paraventricular nucleus elicited a rapid, short decrease in blood pressure[73,74]. Conversely, both infusion of ADM into the intracerebral fluid and micro-injections of ADM in the rostral ventrolateral medulla have been shown to increase blood pressure in animal studies[75,76].

Adrenomedullin regulates endothelial barrier function

The vascular endothelium comprises the inner layer of all blood vessels. This single-cell vascular barrier separates the intravascular from the interstitial space and regulates diffusion of molecules and other substrates through paracellular and transcellular transport[77,78]. Additional roles of the endothelium include regulation of vessel tone, vascular wall permeability, inflammation, hemostasis and angiogenesis[6,78,79]. Inflammation leads to barrier compromise at the level of the endothelial cell-cell junction, causing the boundary between intravascular and interstitial spaces to become more porous, subsequently allowing for leakage of inflammatory mediators (e.g. cytokines and prostaglandins) to the interstitium and leukocyte infiltration into the tissues[6]. This “leaky barrier”

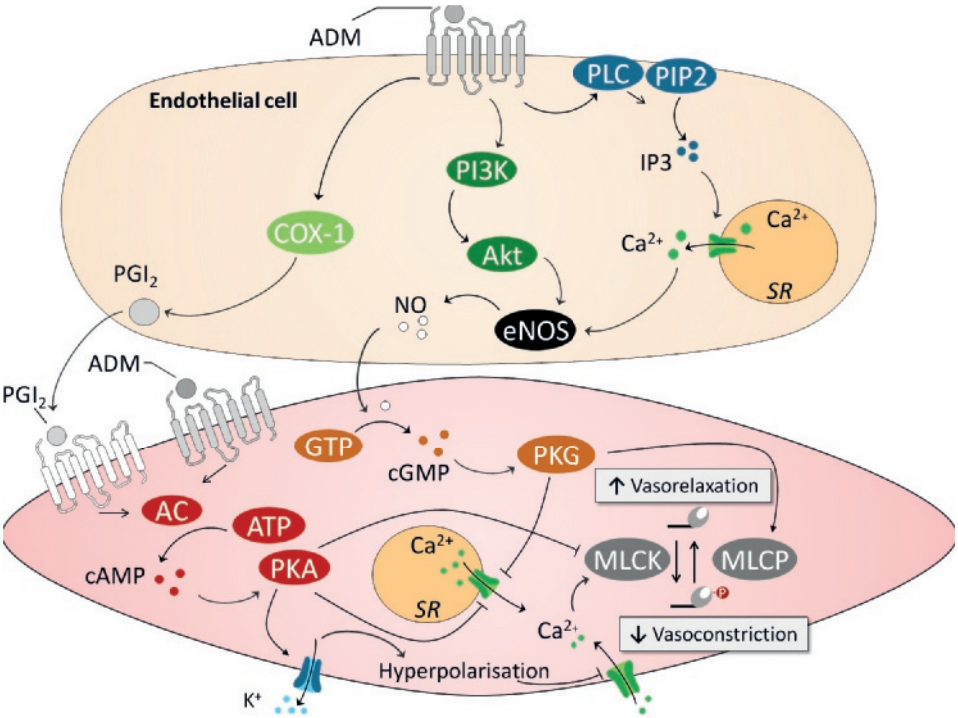

Figure 1. ADM causes vasodilation through endothelium-dependent and endothelium-independent pathways. In an endothelium-independent way, binding of ADM with its receptors on VSMCs increases intracellular cAMP. This leads to subsequent activation PKA which inhibits smooth muscle cell contraction in several ways. First, PKA opens VSMC potassium channels, causing potassium efflux, leading to membrane potential hyperpolarisation and closing of voltage gated calcium channels, reducing intracellular calcium content. Other effects of PKA include inhibition of sarcoplasmatic calcium channel and MLCK. The latter of which is essential for actomyosin contraction. Several endothelium-dependent pathways have been identified. This includes a COX/PGI_2 pathway which activates the cAMP pathway in VSMCs. Other involved endothelium-dependent pathways are PI3k/Akt and PLC/IP3, which both activate eNOS which leads to subsequently activation of a cGMP/cGMP-dependent kinase pathway in VSMCs. This pathway leads to activation of MLCP which 'inactivates' the myosin light chain, and again lowers levels of calcium by inhibiting sarcoplasmatic calcium channels. *Abbreviations: AC, Adenylyl cyclase; ADM, adrenomedullin; AKT, protein kinase B; ATP, adenosine triphosphate; Ca^{2+}, calcium; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; COX-1, cyclooxygenase-1; eNOS; endothelial nitric oxide synthase; GTP, guanosine triphosphate; IP3, inositol triphosphate; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; NO, nitric oxide; PGI_2, prostacyclin; PI3K phosphoinositide 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PLC, phospholipase C; SR, sarcoplasmatic reticulum; VSMC, vascular smooth muscle cell.*

is part of the physiological response to infection, as it is required to combat pathogens in tissues. However, the excessive endothelial barrier disruption observed in sepsis also results in large amounts of fluid leaking from the blood into the tissues, where it accumulates and forms interstitial edema[5,80]. This is a major contributor to the development of shock. Underlying mechanisms of endothelial barrier compromise include rearrangement of the actin cytoskeleton, with cortical actin bundles promoting adherens junction formation and endothelial cell junction tightening, whereas the formation of stress-fibers and phosphorylation of the myosin light chain promotes junction dissociation[81].

ADM is essential for endothelial barrier development and barrier stability. In knock-out models where crucial parts of the ADM-ADM receptor signaling pathway were deleted, development of lethal hydrops fetalis was noted, indicating inadequate development of the endothelial barrier[82,83]. Moreover, in conditional knock-out models, in which either ADM production by endothelial cells (ECs), or formation of the RAMP2 part of the ADM1 receptor was abolished, increased vascular permeability and systemic edema formation was observed[84,85]. This coincided with altered expression of the small GTPases Rac1 (Ras-related C3 botulinum toxin substrate 1) and RhoA (Ras homolog gene family, member A), which are involved in the formation of cortical actin and stress fibers; concentration of the protective GTPase Rac1 were reduced whereas levels of the detrimental RhoA GTPase were increased[85].

Additional preclinical work has elucidated underlying intracellular signaling pathways involved in the endothelial barrier-stabilising effects of ADM. In cultured human umbilical vein endothelial cell (HUVEC) cultures and porcine pulmonary artery endothelial cell (PAEC) monolayers, pretreatment with ADM reduced endothelial hyperpermeability elicited by hydrogen peroxide, thrombin or hemolysin A by attenuating myosin light chain phosphorylation, stress-fiber formation and subsequent gap formation through a cAMP-dependent mechanism[86]. Moreover, ADM pretreatment diminished hydrogen peroxide-induced edema formation in isolated perfused rabbit lungs, which was accompanied by increased cAMP levels in the lung perfusate[86]. Other preclinical work demonstrated similar effects; both treatment with ADM prior to and following an inflammatory insult reduced endothelial hyperpermeability in S. aureus α-toxin-exposed isolated rat ileum, again by reducing endothelial myosin light chain phosphorylation and endothelial

cell contraction[87]. In cortactin-deficient HMEC-1 (human microvascular endothelial cell) monolayers, which show increased permeability, ADM administration reversed myosin light chain phosphorylation and stress-fiber formation through ADM-induced Rap1 activation and Rock1 inhibition[89]. In line, ADM rescued the increase in endothelial permeability in cortactin knockout mice[88]. Similar effects were observed in lymphatic endothelial cells, in which ADM stimulation caused a reorganization of the tight junction protein ZO-1 (zonula occludens-1) and VE-cadherin in the plasma membrane, thereby tightening the membrane[89]. Other experiments demonstrated barrier disrupting effects of ADM blockade through functional inhibition of the VE-cadherin/β-catenin complex[90]. Underlying mechanisms included induction of Src-dependent VE-cadherin phosphorylation, which prevented binding of β-catenin to the cytoplasmic tail of VE-cadherin, inhibiting cell barrier function. Furthermore, β-catenin phosphorylation was induced, which targets β-catenin for ubiquitination and proteasomal degradation. Finally, possible involvement of the PI3K/Akt pathway was suggested[90]. These data emphasise that the ADM system is essential for endothelial barrier stabilization.

Figure 2 summarizes the mechanisms through which ADM may stabilize the endothelial barrier. Note that the cAMP/PKA pathway once again plays an important role. Ligation of ADM with its receptors elicits a strong increase in intracellular cAMP in endothelial cells, which is thought to be one of the most important signaling molecules involved in stabilization of the endothelial barrier[69,91]. This results in subsequent activation of PKA and inhibition of Rho GTPase (i.e. RhoA; Ras homolog gene family, member A). Independent of PKA, cAMP leads to activation of Rap1 by the Rap1 guanine-exchange factor EPAC[81,92]. Rap1 is thought to enhance endothelial cell barrier function in multiple ways, including inhibition of RhoA which reduces actomyosin-induced tension on adherens junctions[81]. Moreover, Rap1 promotes junctional adhesiveness via Afadin, a promoter of junctional tightening by mediating attachment of adherens junctions and the actin cytoskeleton[81]. Finally, both PKA and Rap1 activate Rac1, which results in enforcement of adherens junctions and strengthening of the cortical actin cytoskeleton[93] and inhibition of RhoA[93]. Another relevant mechanism through which ADM exerts barrier-enhancing effects is by stabilizing the VE-cadherin/ β-catenin complex at the cell-cell junctions, possibly mediated through the PI3K/Akt pathway.

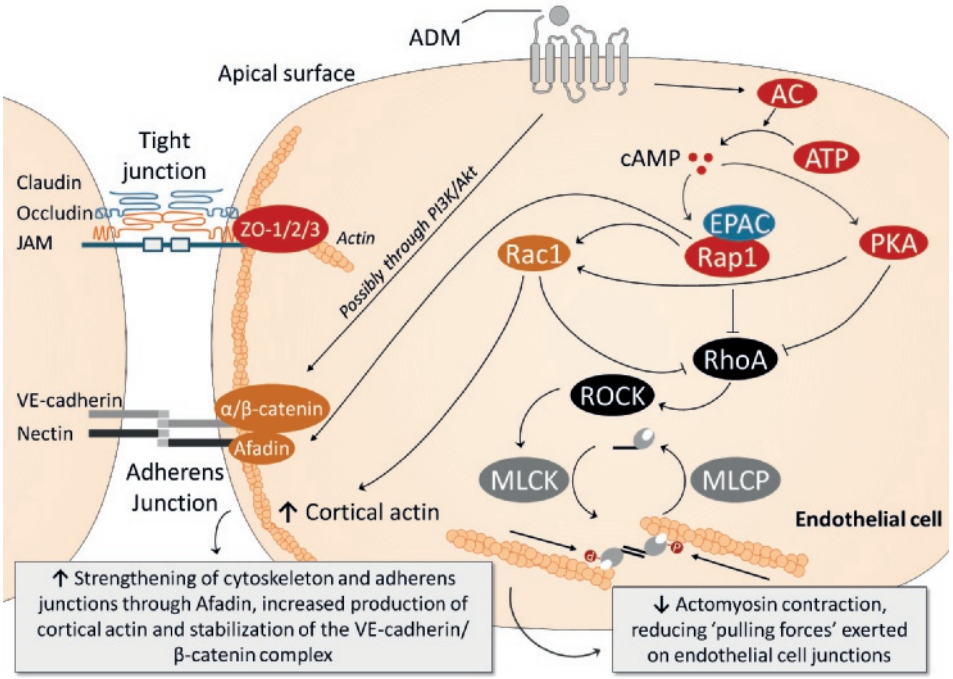

Figure 2. Several pathways have been identified through which ADM exerts endothelial barrier stabilizing effects. Ligation of ADM with its receptors elicits a strong increase in intracellular cAMP in endothelial cells, which subsequently activates PKA and, through activation of EPAC, Rap1. PKA and Rap1 inhibit RhoA/ROCK which results in reduced myosin light chain phosphorylation, decreasing actomyosin contraction (i.e. the 'pulling forces' exerted on the endothelial cell junctions). Rap1 also promotes junctional adhesiveness via Afadin, strengthening junctional tightening by mediating attachment of adherens junctions and the actin cytoskeleton. PKA also increases cortical actin formation through Rac1, which promotes cell-cell stability and cell-matrix adhesion by its connection to tight and adherens junctions. Moreover, Rac1 is also able to inhibit RhoA, decreasing myosin light chain phosphorylation and actomyosin contraction, similar to PKA and Rap1. Ligation of ADM with its receptor is also thought to prevent phosphorylation of VE-cadherin and β-catenin complexes (which would be detrimental for barrier function because phosphorylation of VE-cadherin prevents binding of β-catenin to the cytoplasmic tail of VE-cadherin, and because phosphorylation of β-catenin targets β-catenin for ubiquination and proteasomal degradation), through the PI3K/Akt pathway. *Abbreviations: AC, Adenylyl cyclase; ADM, adrenomedullin; AJ, adherens junction; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; EPAC, exchange factor directly activated by cAMP; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; PI3K/Akt, phosphatidylinositol-4,5-bisphosphate 3-kinase-protein kinase B; PKA, protein kinase A; Rac, Ras-related C3 botulinum toxin substrate 1; Rap1, Ras-related protein 1; ROCK, rho-associated protein kinase; TJ, tight junction; VE-cadherin, vascular endothelial-cadherin; ZO, zonula occludens.*

Miscellaneous effects of adrenomedullin relevant for sepsis

The above described data suggest potential utilization of the ADM system for the treatment of diseases with marked endothelial barrier dysfunction, of which septic shock is a prime example, although these beneficial properties might be offset by vasodilatory effects, an issue we will discuss later on in this review. Furthermore, next to effects on vascular tone and the endothelial barrier, ADM also has other properties relevant in the context of sepsis, including immunoregulatory, anti-microbial and cardioprotective effects.

Immunoregulatory effects

The immune system plays a pivotal role in the pathogenesis of sepsis[4,8]. Therefore, it is relevant to discuss the potential immunoregulatory effects of ADM. Several *in vitro* studies have demonstrated that ADM exerts anti-inflammatory effects and we have summarized the involved pathways in **Figure 3**. One of the first studies conducted on this matter, investigated the effects of ADM in lipopolysaccharide (LPS)-stimulated rat alveolar macrophages. Interestingly, ADM significantly inhibited cytokine-induced neutrophil chemoattractant (CINC/CXCL-1) release, possibly through a cAMP-dependent mechanism[94]. Other experiments in Swiss 3T3 murine fibroblasts demonstrated that ADM inhibits interleukin-1 beta (IL-1β)-induced tumor necrosis factor alpha (TNFα) secretion and confirmed the major role of the cAMP-PKA pathway: A cAMP-dependent protein kinase inhibitor was able to negate ADM's inhibitory effects[95]. Similar effects of ADM were observed in microglia upon stimulation with LPS, inhibiting both TNFα and IL-6[96], as well as in LPS-stimulated murine RAW264.7 macrophages and rat Kupffer cells[97]. *In vivo* experiments have confirmed these *in vitro* studies. Coadministration of ADM and AMBP-1 (adrenomedullin binding protein-1, a protective peptide with putative ADM-enhancing effects) in a rat endotoxemia model attenuated the TNFα response through a mechanism that involves PPARG (peroxisome proliferator-activated receptor-gamma)[98]. Interestingly, ADM has also been a subject of interest for the treatment of inflammatory bowel disease. Intracolonic administration of ADM resulted in a dose-dependent and significant reduction of the size of the ulcerative lesions in a model of acetic acid-induced colitis, and reduced tissue IL-6 levels[99]. Subsequent studies have confirmed these results. For instance, lower levels of IFN-γ (interferon-gamma) and TNFα were observed in rodent models of dextran sulphate sodium-induced colitis[100,101]. A case series on 7 ulcerative colitis patients that received intravenous infusion of ADM for 8 hours daily

over a period of 2 weeks reported improved disease activity index scores, and substantial improvement of ulcers upon endoscopic examination[102]. No serious adverse effects were observed, apart from minor effects on blood pressure and heart rate.

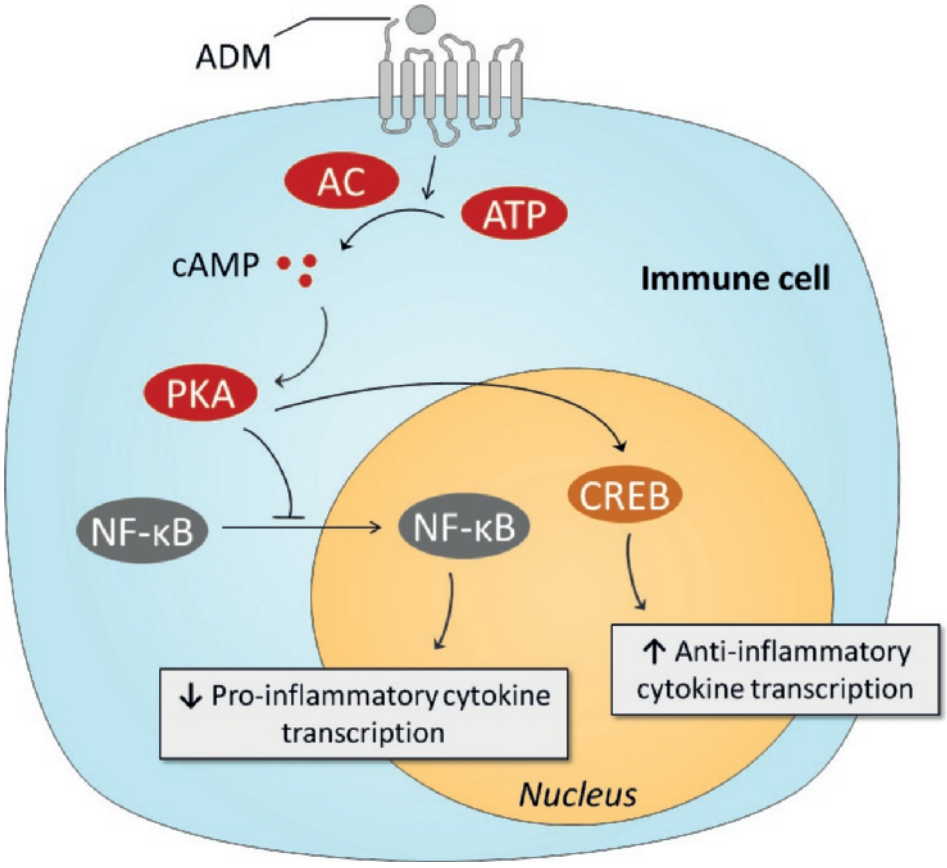

Figure 3. Intracellular mechanisms behind ADM-induced anti-inflammatory effects. Stimulation of the ADM-receptors results in increased intracellular cAMP concentrations, which subsequently activate PKA. PKA prevents NF-κB from entering the nucleus, resulting in reduced transcription of pro-inflammatory genes. PKA-induced activation of CREB results in augmented anti-inflammatory transcription of anti-inflammatory cytokines. *Abbreviations: AC, Adenylyl cyclase; ADM, adrenomedullin; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element-binding protein; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PKA, protein kinase A.*

Anti-microbial properties

The epithelium represents the first protective barrier against pathogens. Many types of epithelial cells secrete ADM, and it can thus be found in many bodily fluids at much higher concentrations than in plasma[103,104]. ADM has chemical and structural similarities with other antimicrobial peptides (i.e. β-defensin-2), including peptide length, a net positive charge, a disulphide bond between residues 16 and 21 and an amidated tyrosine at the carboxyl terminus[105]. This forms an amphipathic structure which permits bacterial membrane intercalation[106]. *In vitro* studies have demonstrated that both the ADM peptide and also smaller ADM fragments are able to inhibit bacterial growth[107].

Cardiac protection

ADM may also confer cardioprotective effects. Increased cardiac hypertrophy and fibrosis were observed after subjecting heterozygous ADM knock-out mice to stress-induced cardiac hypertrophy compared with their wild-type counterparts[108,109]. Other work demonstrated ADM-induced reduction of doxorubicin-induced cardiac myocyte apoptosis via a cAMP-dependent mechanism[110], which was later confirmed *in vivo* in a mice model[111]. The first steps concerning ADM treatment in heart failure patients have been undertaken. In patients with stable congestive heart failure, a short-course ADM infusion resulted in a significant decrease of pulmonary capillary wedge pressure and pulmonary arterial pressure, as well as an increase of cardiac index[58]. Moreover, ADM increased urinary volume and sodium excretion, while decreasing plasma aldosterone levels. In a pilot study in patients with acute decompensated heart failure, combined therapy of ADM and human atrial natriuretic peptide also resulted in beneficial hemodynamic and hormonal changes, including decreased pulmonary arterial pressure, increased urine production and reduced aldosterone and brain natriuretic peptide plasma concentrations[112]. Until now, no further studies have been conducted in patients with heart failure.

In contrast to the data presented above, ADM has also been named a 'cardiac depressant factor', because administration of an ADM-receptor antagonist resulted in increased myocyte contractility in isolated ventricular cardiac myocytes during the early phase rat endotoxemia, although no measurements of cardiac output were performed[113,114].

Adrenomedullin in sepsis

Several processes that take place during sepsis stimulate ADM secretion, including hypoxia, increased circulating levels of LPS, and production of cytokines such as TNF, interleukin-1 (IL-1), and interferon-γ (IFN-γ)[33,115-118]. Circulating ADM levels have been measured in various pathophysiological conditions, and interestingly, the highest concentrations were found in patients with septic shock[119-122]. In sepsis patients, circulating ADM levels correlated with relaxation of vascular tone[123] as well as with disease severity and mortality[119-121,124]. These associations suggest that ADM may play a detrimental role in sepsis, and that ADM-targeted therapies could be of benefit. However, no causal relationships can be deducted from these observational studies and it may also be possible that increases in ADM represent a (failing) compensatory response. In other words, in light of ADM's aforementioned beneficial effects on various pathophysiological processes that take place during sepsis, increased ADM levels might also represent a strategy employed by the body to curtail organ damage during sepsis.

Adrenomedullin and adrenomedullin-targeted therapy as treatment strategies relevant for sepsis

Over the last decades, many have sought to investigate whether administration of ADM, modulation of its function, or antagonizing ADM may influence outcome in various preclinical models of sepsis as well as in models of systemic inflammation and organ injury. Below, we provide an overview of the available data on each of these treatment strategies. Please note that models of systemic inflammation and organ injury do not comprehensively mimic sepsis, but do capture distinct pathophysiological hallmarks of the disease and are therefore of relevance for this overview.

Adrenomedullin administration

An overview of preclinical studies that have investigated the effects of ADM administration is presented in **Table 1**. It needs to be emphasized that except for one, these studies were not performed using infection models, but in clinically less relevant models of systemic inflammation or organ injury.

ADM administration resulted in improved hemodynamics, reduced vascular leakage and organ damage, and improved outcome in different models of endotoxemia[125-129]. Furthermore, beneficial effects of ADM were reported on various outcome measurements in models of lung injury, including attenuated endothelial hyperpermeability, liver injury, less histopathological

changes and reduced pro-inflammatory cytokine levels[130-132]. Moreover, ADM showed protective effects on organ injury in several models of acute kidney injury[133,134]. Although the potential beneficial effects of ADM infusion have been extensively investigated in the abovementioned models of endotoxemia, lung- and renal injury, data obtained in models that are more relevant to sepsis (for example, in resuscitated cecal ligation and puncture models in larger animals) are lacking.

There may be some drawbacks to ADM administration. Because of the short half-life of ADM (22 minutes)[47], infusion would have to be continuous over longer periods of time, as was done previously in ulcerative colitis patients[102]. Moreover, as alluded to before, ADM has potent vasodilatory effects, which may raise concerns for ADM-induced hypotension. Finally, ADM may be difficult to handle in clinical practice, because of its adhesiveness, arguably sticking to artificial surfaces[135].

Table 1. Overview of preclinical studies investigating ADM administration in different models related to sepsis.

Intervention	Model	Results (compared to placebo)	Reference
Bolus of ADM (pretreatment)	H_2O_2-induced vascular leakage in isolated, mechanically ventilated rabbit lungs	↓ Vascular leakage	Hippenstiel et al.[86]
Continuous infusion of incremental dosages of ADM (posttreatment)	Ovine endotoxemia (24 hours of Salmonella typhosa LPS administration)	↓ Pulmonary vascular resistance ↑ Cardiac index and heart rate	Westphal et al.[128]
Continuous infusion of ADM (pre- and posttreatment groups)	Isolated rat ileum with S. aureus α-toxin administration	↑ Endothelial barrier function in both pre- and posttreatment groups.	Brell et al.[87]
Continuous infusion of ADM (pre- and posttreatment groups)	Ovine endotoxemia (4 hours of Salmonella typhosa LPS administration)	↑ Cardiac index ↓ Pulmonary arterial pressure ↑ Lactate clearance (for both pre- and posttreatment groups)	Ertmer et al.[125]
Continuous infusion of ADM (pretreatment)	Intratracheal LPS-induced lung injury in rats	↓ Vascular leakage ↓ Histopathological abnormalities ↓ Protein, albumin and inflammatory markers in BAL fluid	Itoh et al.[126]
Continuous infusion of ADM (posttreatment)	S. aureus α-toxin model in resuscitated rats.	↓ Vascular leakage ↑ 6-hour blood pressure ↑ Cardiac index and 6-hour survival	Temmesfeld-Wollbrück et al.[127]
Continuous infusion of ADM (posttreatment)	S. aureus α-toxin model in rats	↓ Gut epithelial hyperpermeability	Temmesfeld-Wollbrück et al.[129]
Continuous infusion of ADM (started pretreatment in 2 hour experiments, and after 2 hours of ventilation in the 6 hour experiments)	Ventilator induced lung injury model in mice, experiments with 2 and 6 hours of ventilation	For both experiments ↓ Lung hypermeability, leukocyte accumulation and MLCP expression ↑ Oxygenation, lactate and creatinine clearance	Müller et al.[130]
Bolus of intrapleural ADM (posttreatment)	Carrageenan-induced pleurisy model in mice	↓ Pro-inflammatory cytokines ↓ Oxidative and nitroxidative lung tissue injury	Talero et al.[132]
Continuous infusion of ADM (pretreatment)	Aortic ischemia-reperfusion in rats	↓ Kidney injury (various morphological and biochemical parameters)	Oyar et al.[134]
Bolus of ADM (pretreatment)	Contrast-induced nephropathy in rats	↓ Kidney injury and inflammation	Inal et al.[133]
Continuous infusion of ADM (pretreatment)	Pneumococcal pneumonia in mechanically ventilated mice	↓ Lung injury ↓ Lung hyperpermeability ↓ Indirect liver and gut injury	Müller-Redetzky et al.[131]

Only studies using models of sepsis or models that capture some of the prominent hallmarks of sepsis have been included. *Abbreviations: ADM, adrenomedullin; BAL, bronchoalveolar lavage; E. coli, Escherichia coli; H_2O_2, hydrogen peroxide; LPS, lipopolysaccharide; MLCP, myosin light chain phosphorylase; S. aureus; Staphylococcus aureus.*

Coadministration of adrenomedullin and complement factor H

Complement factor H is thought to be capable of binding to ADM (and therefore also known as 'ADM binding protein-1' [AMBP-1] in this context), and chaperone ADM in the circulation[136]. However, note that this has been subject to some debate in literature. The observed *in vitro* binding could theoretically be due to unspecific (ionic) interaction. Interference of complement factor H with ADM could not be demonstrated in other recent work, in which up to almost 400.000 fold molar excess of complement factor H did not influence ADM recovery[137]. Furthermore, *in vivo* plasma levels of complement factor H are approximately 10^9 fold higher than ADM levels. Therefore, it could be hypothesised that exogenous administered complement factor H would not add significantly to endogenous levels.

The ADM binding site of AMBP-1 has not yet been discovered, although it is thought that AMBP-1 may modulate ADM activity and degradation. A functional assay revealed a 2-fold increased cAMP response after coincubation of cells with ADM and AMBP-1 compared to incubation with ADM alone[138]. Other work has demonstrated that AMBP-1 protects ADM from proteolytic degradation[49], thereby presumably increasing its half-life. Because of these possible potentiating effects, several studies have investigated the therapeutic potential of coadministration of ADM and AMBP-1 in various preclinical animal models. Initially, the effects of coadministration of ADM and AMBP-1 were assessed in a model of CLP-induced sepsis in rats, where pretreatment with the combination of ADM and AMBP-1, but not of each compound individually, resulted in positive effects on hemodynamic parameters, augmenting oxygen delivery, cardiac output and lactate clearance[139]. Furthermore, improved 10-day survival was observed in animals undergoing CLP surgery. Note that in these survival experiments, treatment was started 5 hours after CLP surgery. Other studies have also demonstrated beneficial effects coadministration of ADM and AMBP-1 in models of hemorrhagic shock[140-142], ischemia/reperfusion[143,144], endotoxemia[98] and septic shock[145,146]. To what extent complement factor H influences the described ADM-mediated effects remains unclear as the majority of preclinical studies on ADM with AMBP-1 coadministration did not compare ADM/AMBP-1 with ADM alone. Please refer to **Table 2** for an overview of this work.

Table 2. Overview of preclinical studies investigating ADM with co-administration of AMBP-1 in different models related to sepsis.

Intervention	Model	Results (compared to placebo)	Reference
ADM and AMBP-1 (posttreatment)	Cecal ligation and puncture (CLP) induced sepsis in rats	↑ CO, DO_2 and lactate clearance ↑ Hepatic blood flow ↓ Plasma ALT, AST ↓ Hemodilution ↑ 10-day survival	Yang et al.[139]
ADM and AMBP-1 for 45 min (posttreatment)	Hemorrhagic shock rats (MAP 40 mmHg for 90 min), then resuscitated	↓ Plasma AST, ALT, TNF and HMGB-1 ↑ IL-10 ↑ Lactate and creatinine clearance ↑ 12-day survival	Cui et al.[140]
ADM and AMBP-1 (posttreatment)	Hemorrhagic shock rats (MAP 40 mmHg for 90 min), then resuscitated	↑ CO and organ blood flow (liver, kidney and small intestine) ↓ Cardiac TNF-α	Wu et al.[142]
ADM and AMBP-1 at start of reperfusion	Intestinal ischemia-reperfusion in rats	↓ Plasma TNF-α, IL-1β, IL-6 and IL-10 ↓ Plasma AST and ALAT ↓ Histopathological changes small intestine ↑ Lactate and creatinine clearance ↑ 10-day survival	Carrizo et al.[144]
ADM and AMBP-1 (pretreatment)	Endotoxemic rats	↓ TNF-α ↑ IL-10 ↑ Lactate clearance	Miksa et al.[98]
ADM and AMBP-1 (pretreatment)	CLP-induced sepsis in rats	↑ eNOS signaling ↓ Endothelial dysfunction	Zhou et al.[156]
ADM and AMBP-1 at start of reperfusion	Intestinal ischemia-reperfusion induced lung injury in rats	↓ Lung edema ↓ Lung TNF-α and IL-6 ↓ Histopathological changes	Dwivedi et al.[143]
ADM and AMBP-1 (posttreatment)	Hemorrhagic rats (MAP 40 mmHg for 90 min), then resuscitated	↓ Plasma AST and ALT ↑ Lactate and creatinine clearance ↓ Plasma TNF-α and IL-6 ↑ 12-day survival	Wu et al.[141]
ADM and AMBP-1 at start of reperfusion	Renal ischemia-reperfusion in rats	↓ Renal edema ↓ Tissue injury ↓ Plasma and tissue pro-inflammatory cytokines	Shah et al.[157]
ADM and AMBP-1 (posttreatment)	Bile duct ligation / CLP model of induced sepsis in rats	↓ Systemic markers of tissue injury ↓ Inflammatory response ↑ 7-day survival	Yang et al.[146]

Only studies using models of sepsis or models that capture some of the prominent hallmarks of sepsis have been included. *Abbreviations: Adrenomedullin, ADM; ALT, alanine aminotransferase; AMBP-1, adrenomedullin binding protein-1; AST, aspartate aminotransferase; BAL, bronchoalveolar lavage; CLP, cecal ligation and puncture; CO, cardiac output; DO_2, delivery of oxygen rate; E. coli, Escherichia coli; H_2O_2, hydrogen peroxide; HMGB-1, high mobility group box protein 1; IL, interleukin; LPS, lipopolysaccharide; MLCP, myosin light chain phosphorylase; S. aureus; Staphylococcus aureus; TNF, tumor necrosis factor.*

Antibodies against and/or receptor antagonists of adrenomedullin

To date, three studies have investigated the effects of ADM antagonists on hemodynamic parameters in preclinical models of endotoxemia and sepsis, using either a neutralizing anti-ADM antibody[147] or the ADM receptor antagonist ADM(22-52)[113,148]. Both these treatments prevented the occurrence of a 'hyperdynamic' hemodynamic response (characterized by decreased blood pressure and peripheral vascular resistance, and an increased cardiac output) during the first hours after induction of sepsis or systemic inflammation[147,148]. This is in line with previous data demonstrating vasodilatory effects of ADM, accompanied by a reduction of peripheral vascular resistance and increase of cardiac output. Furthermore, ADM(22-52) administration after initiation of endotoxemia resulted in improved myocyte contractility, but did not improve 7-day survival[113].

Further efforts have been put into the development of various high-affinity monoclonal anti-ADM antibodies, each targeting different regions of the ADM peptide, resulting in full or partial inhibition of ADM signalling. The efficacy of these antibodies was investigated in a survival study in CLP-induced sepsis in mice[149]. A non-neutralizing antibody targeted against the N-terminus of ADM, which only partially inhibits ADM-signalling, conferred survival benefit, whereas a completely inhibiting antibody targeted against the C-terminal, did not. Subsequent experiments were conducted in a model of resuscitated CLP-induced murine sepsis, in which pretreatment with the non-neutralizing antibody resulted in decreased catecholamine infusion rates, kidney dysfunction, iNOS, but not eNOS expression, and ultimately improved survival[150]. Due to these positive results, a humanized version of the antibody, named Adrecizumab, has been developed for further clinical development. Beneficial effects of Adrecizumab on vascular barrier function and survival were recently demonstrated in preclinical models of systemic inflammation and sepsis[151]. In this study, pretreatment with Adrecizumab attenuated renal vascular leakage in endotoxemic rats as well as in mice with CLP-induced sepsis, which coincided with increased renal expression of the protective peptide Ang-1 and reduced expression of the detrimental peptide VEGF[151]. Also, pretreatment with Adrecizumab improved 7-day survival in CLP-induced sepsis in mice from 10% to 50% for single and 0% to 40% for repeated dose administration[151]. Moreover, in a phase I study excellent safety and tolerability was demonstrated: no serious adverse events were observed, no signal of adverse events occurring more frequently in Adrecizumab-treated

subjects was detected, and no relevant changes in other safety parameters were found[152]. Of particular interest is the proposed mechanism of action of Adrecizumab. Both animal and human data reveal a potent, dose-dependent increase of circulating ADM following administration of this antibody. Based on pharmacokinetic data and the lack of an increase in MR-proADM (an inactive peptide fragment derived from the same prohormone as ADM), the higher circulating ADM levels cannot be explained by an increased production[152]. A mechanistic explanation for this increase could be that the excess of antibody in the circulation may drain ADM from the interstitium to the circulation, since ADM is small enough to cross the endothelial barrier, whereas the antibody is not. Additionally, binding of the antibody to ADM leads to a prolongation of ADM's half-life[153]. Even though Adrecizumab partially inhibits ADM signaling, a large increase of circulating ADM results in an overall 'net' increase of ADM activity in the blood compartment, where it exerts beneficial effects on endothelial cells (predominantly barrier stabilization), whereas ADM's detrimental effects on VSMCs (vasodilation) in the interstitium are reduced[153]. This hypothesis is in line with previous studies demonstrating overall beneficial effects of agonists of the ADM system, whereas complete inhibition of ADM was shown not to improve outcome. A detailed description of the proposed mechanisms of action of Adrecizumab is provided elsewhere[153]. Please refer to **Table 3** for an overview of studies that investigated ADM-antagonists and/or modulating antibodies. Currently, a phase II study with Adrecizumab is ongoing in septic patients (clinicaltrials.gov identifier: NCT03085758).

PEGylation of adrenomedullin

PEGylation is the process by which polyethylene glycol (PEG) chains are attached to protein and peptide drugs[154]. PEGylation of polypeptide drugs often results in improved pharmacokinetic and pharmacodynamic properties, as it offers protection from proteolytic enzymes, increases water solubility, reduces renal clearance, and limits toxicity[154]. Human ADM was previously molecularly modified by conjugating ADM's N-terminal with PEG, in an attempt to reduce potentially unfavourable effects of ADM (hypotension, activated sympathetic nerve activity, and increased renin secretion)[156]. Compared to native ADM, PEGylated ADM had a slightly lower half maximal effective concentration (EC_{50}) in a functional assay, while the maximum possible effect (E_{max}) values remained similar. Moreover, in rats, PEGylated ADM resulted in a longer half-life and a significantly less blood lowering

effect compared with native ADM[155]. A subsequent study in a mouse DSS-induced colitis model revealed an attenuation of the total inflammation score. Unfortunately, no studies have been performed in animal sepsis models.

Table 3. Overview of preclinical studies with adrenomedullin antibodies and/or antagonists in different models related to sepsis.

Intervention	Model	Results	Reference
Anti-ADM antibody (post-treatment)	CLP-induced sepsis in rats	Anti-ADM antibodies prevented occurrence of hyperdynamic response during first 5 hrs after CLP	Wang et al.[147]
ADM receptor antagonist ADM(22-52) (pretreatment)	E. coli LPS in rats	↑ Blood pressure (during first 6 hours)	Mazzocchi et al.[148]
ADM antagonist ADM(22-52) (posttreatment)	E. coli LPS in rats	↑ Survival myocyte contractility No effect on 7-day survival	Hyvelin et al.[113]
N-terminus murine antibody against N-terminus of ADM (pretreatment)	CLP induced sepsis in mice	↑ Survival	Struck et al.[149]
N-terminus murine antibody against N-terminus of ADM (pretreatment)	Resuscitated CLP-induced sepsis in mice	↓ Noradrenaline infusion rates ↑ Urine production, ↑ Creatinine clearance & ↓NGAL ↓iNOS and peroxynitrate formation ↓ Systemic inflammation ↓ Tissue apoptosis	Wagner et al.[150]
N-terminus humanized antibody against N-terminus of ADM (pretreatment)	E. coli LPS in rats CLP induced sepsis in mice	↓ Vascular leakage in LPS rats ↓ Renal vascular leakage, ↓ VEGF and ↑ angiopoietin-1 levels in CLP mice ↑ Survival in CLP mice	Geven et al.[151]

Only studies using models of sepsis or models that capture some of the prominent hallmarks of sepsis have been included. *Abbreviations: ADM, adrenomedullin; CLP, cecal ligation and puncture; E. coli, Escherichia coli; iNOS, inducable nitric oxide synthase; LPS, lipopolysaccharide; VEGF, vascular endothelial growth factor.*

Conclusion

Adrenomedullin is an important peptide hormone involved in sepsis. Its effects include vasodilation, stabilization of the endothelial barrier and immunoregulation. Administration of ADM in animal models of inflammation, organ injury and infection resulted in improved outcome. Attempts have been made to negate the potential hypotensive effects of ADM to further enhance its beneficial effects. Coadministration of ADM with ADM binding peptide-1, administration of ADM bound to polyethylene glycol and administration of partially inhibiting ADM antibodies (which in fact increase the net circulating ADM levels without causing hypotension) showed promising results. However, it is difficult to translate these results to septic patients, because these preclinical studies have often been performed in small animals using clinically less relevant models of systemic inflammation or induced organ injury. Moreover, in a significant proportion of studies no resuscitation or antibiotics were applied, and the intervention was initiated prior to the induction of disease. Finally, many treatments have not been compared head-to-head. Given the current lack of adjuvant therapies in sepsis, future research on this promising peptide in more relevant animal models of sepsis and ultimately humans is highly warranted.

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Chapter 3

Vascular effects of adrenomedullin and the anti-adrenomedullin antibody Adrecizumab in sepsis

Christopher Geven, Andreas Bergmann, Matthijs Kox and Peter Pickkers

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Abstract

Sepsis remains a major scientific and medical challenge, for which, apart from significant refinements in supportive therapy, treatment has barely changed over the last few decades. During sepsis, both vascular tone and vascular integrity are compromised, and contribute to the development of shock. The free circulating peptide adrenomedullin (ADM) is involved in the regulation of the endothelial barrier function and tone of blood vessels. Several animal studies have shown that ADM administration improves outcome of sepsis. However, in higher dosages, ADM administration may cause hypotension, limiting its clinical applicability. Moreover, ADM has a very short half-life and easily adheres to surfaces, further hampering its clinical use. The non-neutralizing anti-ADM antibody Adrecizumab (HAM8101) which causes a long lasting increase of plasma ADM has shown promising results in animal models of systemic inflammation and sepsis; it reduced inflammation, attenuated vascular leakage, and improved hemodynamics, kidney function, and survival. Combined with an excellent safety profile derived from animal and phase I human studies, Adrecizumab represents a promising candidate drug for the adjunctive treatment of sepsis. In this review, we first provide a brief overview of the currently available data on the role of adrenomedullin in sepsis and describe its effects on endothelial barrier function and vasodilation. Furthermore, we provide a novel hypothesis concerning the mechanisms of action through which Adrecizumab may exert its beneficial effects in sepsis.

Introduction

Sepsis is an inflammatory disorder, in which a dysregulated host response to an infection results in life-threatening organ dysfunction[1]. Sepsis is a prevalent syndrome; approximately one third of all patients in Intensive Care Units (ICU) present with sepsis on admission or develop sepsis during their ICU stay[2]. Despite many advances in medical care, the incidence of sepsis is increasing and its mortality remains high[3]. Sepsis is characterized by a complex and multi-layered pathogenesis. In short, a host response is mounted by ligation of pathogen associated molecular patterns (PAMPs) to pattern recognition receptors (PRRs) present on immune cells, in turn activating inflammatory and coagulation pathways, characterized by leukocyte and complement activation as well as release of cytokines, reactive oxygen species, and damage-associated molecular patterns (DAMPs). These events result in perpetuation of the inflammatory response and ultimately cause organ failure, which is a key determinant of survival[4]. The vascular endothelium is a protective barrier involved in the maintenance of vessel integrity that controls diffusion of molecules and fluids between the intravascular and interstitial space. Endothelial function is often compromised during sepsis, leading to increased leukocyte adhesion, vascular wall permeability, and vasodilation[4-7]. These changes result in, amongst others, extravascular fluid accumulation, causing tissue edema, a decrease in circulating volume, hypotension, and subsequent organ failure.

A considerable proportion of deaths are attributed to the early phase of sepsis, as a result of multi-organ failure despite supportive therapy. For decades, treatment of sepsis consists of antimicrobial therapy, source control and supportive treatments such as fluid resuscitation, vasopressor use and mechanical ventilation[6]. Many trials have investigated possible adjuvant treatments, primarily focussing on anti-inflammatory therapies. Unfortunately, not a single intervention is currently in use because of lack of efficacy. This can partially be explained by methodological issues, such as heterogeneity of the study population and timing of the intervention, but also by the complexity of the immune response with multiple pathways contributing to injury[6,8]. Thus, there is still a great unmet need for novel adjuvant therapies for sepsis. Interestingly, interventions aimed at improving endothelial barrier function have only sparsely been investigated, while this represents a highly relevant target[9].

Adrenomedullin

Adrenomedullin (ADM) is a free circulating 52 amino-acid peptide belonging to the calcitonin gene-related peptide family that was first discovered in human pheochromocytoma tissue more than two decades ago[10,11]. Although ADM was initially thought to primarily possess vasodilatory properties[12], it was subsequently discovered that it exerts a multitude of biological effects, both in health and disease, including anti-inflammatory effects, stabilization of endothelial barrier function, and regulation of vascular tone[13]. These effects are attained through binding of ADM to heterodimeric receptor complexes consisting of the calcitonin receptor-like receptor (CRLR) and a specific receptor activity-modifying protein (RAMP), RAMP2 and RAMP3[14]. ADM is produced by many cells, including endothelial cells, vascular smooth muscle cells (VSMCs), monocytes, renal parenchymal cells and macrophages[15-20]. A wide variety of mediators involved in the pathophysiology of sepsis have been reported to enhance ADM production. For instance, in rat VSMCs, interleukin-1 (IL-1) alpha and beta, tumor necrosis factor (TNF) alpha & beta, epinephrine, substance P, endothelin-1, angiotensin II, fetal calf serum (FCS), and lipopolysaccharide (LPS) all increased ADM gene expression and synthesis[21,22]. Conversely, other substances known to play a role in sepsis, such as thrombin, vasoactive intestinal polypeptide and interferon-gamma (IFN-ϒ), were shown to decrease ADM production in VSMCs[22]. Studies in rat endothelial cells revealed that ADM is not stored, but rather constitutively produced, and that endothelial cells secrete ADM at a higher rate than VSMCs[23]. Similar to rat VSMCs, TNF, IL-1, LPS and thyroid hormone increase ADM secretion by endothelial cells, whereas transforming growth factor β1, IFN-ϒ and FCS suppress ADM secretion. Other studies have demonstrated increased ADM production by renal parenchymal cells and endothelial cells under hypoxic conditions[19,24]. The effects of endotoxin and hypoxia on ADM production were also confirmed in vivo in a rat model of LPS-induced inflammation, as well in models of induced hypoxia with inhalation of carbon monoxide and air with reduced concentrations of oxygen[25,26]. ADM has a short circulating half-life (22 minutes)[27], and is removed from the circulation in two ways. First, the ADM receptors function as clearance receptors: upon ligation of ADM with its receptors, the ADM-receptor complex is internalized and subsequently degraded[28]. The lung has been reported as a significant site of clearance via this mechanism[29,30]. The second clearance mechanism is represented by proteolytic degradation of ADM[31-33].

Adrenomedullin in sepsis

Increased levels of circulating ADM are found during sepsis which correlate with relaxation of vascular tone[34] as well as with disease severity and mortality in septic patients[35-38]. Although these associations may suggest that ADM plays a detrimental role in sepsis, they should be interpreted with caution, as no causal relationships – detrimental or beneficial – can be directly deducted from these observational studies. In fact, many experimental studies, both *in vitro*[39] and *in vivo*[40-42], have demonstrated that ADM administration is beneficial during systemic inflammation and sepsis through favorable effects on endothelial barrier function. Another relevant peptide in this context is adrenomedullin-binding protein 1 (AMBP-1), also known as complement factor H[43]. AMBP-1 binds to ADM, albeit with a lower overall affinity than ADM binding to its receptors[44]. AMBP-1 is reported to protect ADM from proteolytic degradation[32], and modulates ADM activity: coincubation of fibroblasts with ADM and AMBP-1 resulted in a 2-fold increase of cyclic adenosine monophosphate (cAMP), compared to treatment with ADM alone[45]. Furthermore, several studies have demonstrated that co-administration of ADM and AMBP-1 improves outcome in preclinical animal studies employing various models of systemic inflammation, organ injury, and septic shock[46-49].

Adrenomedullin stabilizes the endothelial barrier

Studies in genetically modified mice deficient for crucial parts of the ADM receptor signalling pathway reported development of lethal hydrops fetalis, indicating impaired development of the endothelial barrier[50-52]. In conditional murine knock-out models, in which either ADM or the RAMP2 part of the AM1 receptor was abolished in endothelial cells, increased vascular permeability and edema formation was observed[53,54]. Furthermore, *in vitro* studies demonstrated that ADM stabilizes the endothelial barrier through regulation of the actin myosin cytoskeleton[55]. ADM is thought to prevent formation of stress fibers that pull on cell-cell junctions[56], which is primarily mediated through the cAMP-PKA pathway involving Rap1 activation and RhoA/ROCK inhibition[57]. This is accompanied by attenuated endothelial myosin light chain phosphorylation, actomyosin contractility, and ultimately attenuation of endothelial cell gap formation[39,56]. In addition, ADM activates Rac1, which stimulates cortical actin formation[58]. Moreover, completely blocking ADM has been shown to increase endothelial layer permeability by inhibiting cell-cell contacts predominantly through disruption of VE-

cadherin/β-catenin & Akt signalling pathways[59]. **Figure 1** presents a schematic overview of the pathways through which ADM exerts endothelial stabilizing and vasodilatory effects. Beneficial effects of ADM administration on endothelial barrier function have been demonstrated in animals *in vivo*, using models of systemic inflammation and sepsis. For example, ADM administration in a murine model of lung injury resulted in attenuation of pulmonary hyperpermeability, lung injury, and systemic hyperinflammation[42]. In rats, administration of ADM significantly reduced extravasation of albumin and plasma fluid, and ultimately improved survival in septic shock induced by Staphylococcus aureus alpha-toxin[40].

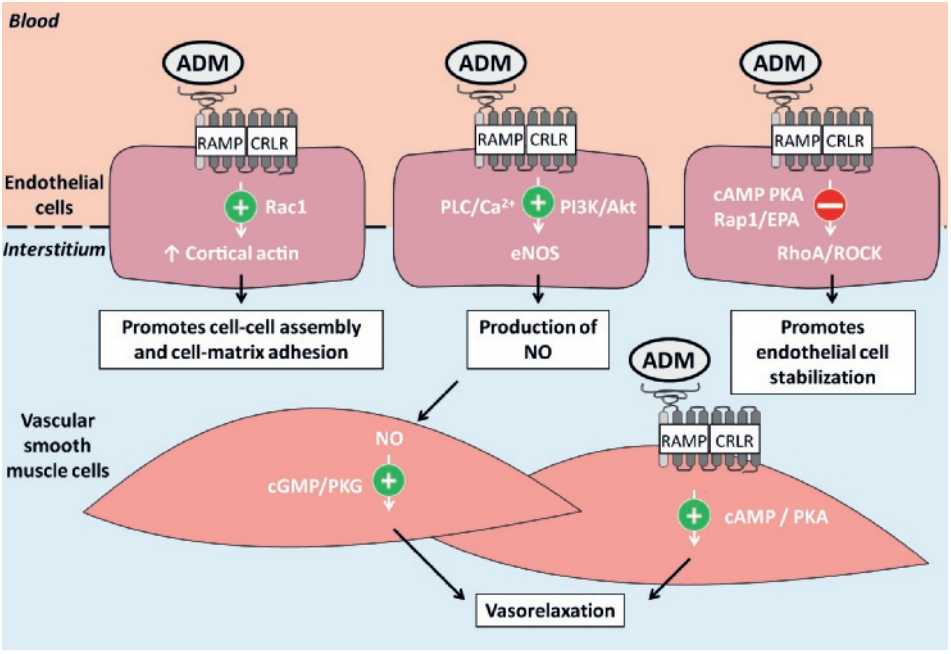

Figure 1. Schematic overview of intracellular pathways through which ADM exerts vasodilatory and endothelial stabilizing effects.

Adrenomedullin exerts vasodilatory effects, has a short half-life, and is difficult to handle

As mentioned earlier, one of the initially discovered properties of ADM is that it lowers blood pressure. Several studies have demonstrated that ADM promotes vasodilation in isolated blood vessels[60,61]. Furthermore, infusion of high dosages of ADM was shown to decrease blood pressure and peripheral vascular resistance in rats, cats, sheep and humans, thereby inducing a compensatory increased heart rate and cardiac output[27,41,62-65]. The vasodilatory

effects of ADM are mediated through binding to the ADM receptors present on vascular endothelial cells and VSMCs. Several signalling pathways, both endothelium-dependent and -independent, appear to account for ADM-mediated vasodilation[66]. Binding of ADM to its receptors on VSMCs leads to increased cAMP[67,68] in an endothelium-independent fashion, which subsequently activates protein kinase A (PKA). PKA phosphorylates a large number of proteins which ultimately results in relaxation of smooth muscle cells. Endothelium-dependent pathways have in common that they act through stimulation of endothelial NO synthase (eNOS), which in turn stimulates the cyclic guanosyl monophosphate (cGMP) system in VSMCs, leading to activation of PKA and protein kinase G (PKG). PKG acts downstream to reduce intracellular calcium levels and alters sensitivity of contractile proteins for calcium, thereby also inducing smooth muscle relaxation. These pathways include the inositol-1,4,5-triphosphate (IP_3) system and the phosphatidylinositol-4,5-bisphosphate 3-kinase-protein kinase B (IP3K/Akt) pathway[69,70]. It remains unknown as to what extent endothelium-dependent or -independent pathways account for ADMs vasodilatory effects.
So, despite ADMs potent endothelial barrier-stabilizing effects, systemic administration in higher dosages exerts hemodynamic effects, mainly vasodilatory, that may be detrimental for sepsis patients and even more so for septic shock patients[71]. Moreover, it has a very short half-life (several minutes)[27,29] and is cumbersome to handle being very adhesive[72] and thus arguably sticking to artificial surfaces used in the clinic. Taken together, these unfavourable pharmacological properties might seriously hamper its clinical applicability in sepsis, and modulating the endogenous ADM response may represent a more viable strategy. Although at first glance it may appear counterintuitive, a specific type of antibody targeting ADM may represent such a strategy.

The anti-adrenomedullin antibody Adrecizumab improves outcome in experimental sepsis

ADM interacts with its receptor via the C-terminal moiety[73], and the N-terminal part of ADM is not required for its agonist function[74]. Previously, several high-affinity mouse monoclonal anti-ADM antibodies were investigated, targeting different epitopes of ADM[75]. Complete functional inhibition of ADM (by blocking the C-terminus) did not improve survival in cecal ligation and puncture (CLP) induced murine sepsis. However, pretreatment with high-affinity antibodies targeting the N-terminus of ADM (HAM1101), which

only partially inhibits ADM signaling, even when applied in large molar excess over ADM, showed profound beneficial effects on mortality[75]. A later study, investigating the effects of this antibody in a resuscitated CLP-induced murine sepsis, reported decreased catecholamine infusion rates, prevention of kidney dysfunction, reduction of iNOS but not eNOS expression, and ultimately improved survival[76]. Following these initial preclinical experiments HAM1101 was humanized to HAM8101 for further preclinical and clinical studies and named Adrecizumab. Adrecizumab was shown to reduce renal interstitial edema in endotoxemic rats and decreased renal VEGF expression (a potent inducer of vascular permeability) in CLP-induced murine sepsis, whereas expression of the protective peptide angiopoietin-1 was augmented[77,78]. Furthermore, it reduced mortality in CLP-induced murine sepsis to a similar extent as the parent antibody HAM1101. An overview of these preclinical antibody studies is provided in **Table 1**.

Table 1. Overview of studies that have investigated Adrecizumab or its murine predecessor.

Authors (year)	Intervention	Model	Effects
Struck (2013)	Pretreatment with murine antibody against N-terminus ADM (HAM1101, 2 mg/kg)	Cecal ligation and puncture (CLP) induced murine sepsis	↑ Survival
Wagner (2013)	N-terminus murine antibody against N-terminus ADM (HAM1101, 2 mg/kg), administered immediately after CLP surgery	Resuscitated CLP-induced murine sepsis	↓ Norepinephrine infusion ↑ Urine production & creatinine clearance ↓ Neutrophil gelatinase-associated lipocalin (NGAL) ↓ iNOS expression & peroxynitrite formation in kidney & aorta ↓ Systemic inflammation ↓ Tissue apoptosis in kidney
Geven (2018)	Pretreatment with humanized antibody against N-terminus ADM (Adrecizumab, 0.02, 0.1, 0.5 or 2.5 mg/kg)	Lipopolysaccharide (LPS) induced systemic inflammation in rats	↓ Renal albumin extravasation
Geven (2018)	Pretreatment Adrecizumab (0.1, 2.0 or 20 mg/kg)	CLP-induced murine sepsis	↓ Renal albumin extravasation ↓ Renal vascular endothelial growth factor (VEGF) ↑ Renal angiopoietin-1
Geven (2018)	Pretreatment single dose Adrecizumab (2 mg/kg) and repeated dose Adrecizumab (pretreatment with 4 mg/kg followed by 2 mg/kg after 24 and 48 hours).	CLP-induced murine sepsis	↑ Survival

Proposed mechanism of action of Adrecizumab

The mechanisms by which Adrecizumab exerts its beneficial effects remain to be fully elucidated. However, there are indications that it involves modulation of the ADM equilibrium between the blood compartment and interstitium. The endothelium is a single cell monolayer, separating the blood compartment from the interstitial space. As ADM is a small peptide (6 kDa), it freely diffuses between the blood vessels and the interstitial space. In this homeostatic 'normal' situation, ADM regulates the endothelial barrier function through ligation of receptors on endothelial cells, and regulates vascular tone through binding to receptors on endothelial cells (indirect effect) and VSMCs (direct effect [**Figure 2A**]). During septic shock, circulating ADM levels are profoundly increased[35,38,79]. Although there is no data on ADM concentrations in the interstitial compartment, it is plausible to assume that ADM concentrations are in equilibrium with the circulation, because ADM is able to diffuse freely between the two compartments[29]. The previously described preclinical studies suggest that ADM present in the circulation counteracts – albeit insufficiently – sepsis-induced vascular leakage through its effects on endothelial cells, while excess circulating and interstitial ADM may lead to vasodilation and septic shock through indirect effects on endothelial and direct effects on VSMCs (**Figure 2B**). Interestingly, in both preclinical studies in healthy animals and animals with LPS-induced inflammation and CLP-induced sepsis, and the recently performed phase I study in humans (unpublished, discussed in the next section), an immediate (<5 minutes) dose-dependent increase of plasma ADM concentrations was observed after Adrecizumab administration[80]. This effect has been observed across all species investigated (mice, rats, beagle dogs, cynomolgus monkeys and humans). The measured elevated plasma ADM concentrations in fact mainly represent complexes of ADM bound to Adrecizumab, as Adrecizumab is administered in several orders of magnitude molar excess over endogenous ADM, and the assay used to measure plasma ADM (sphingotest® bio-ADM, Sphingotec GmbH, Hennigsdorf, Germany) detects both free ADM and ADM complexed with Adrecizumab[81]. Given the small volume of distribution of Adrecizumab (100 mL/kg; unpublished data from phase I study in human volunteers), it can be concluded that Adrecizumab does not freely diffuse from the circulation into the interstitial space, which is not surprising it being a large IgG antibody of 160 kDa. Because plasma concentrations of MR-proADM, a non-functional peptide stemming from the same precursor peptide as ADM do not increase upon administration of Adrecizumab[80], it can be concluded that the antibody

does not induce increased expression of ADM. Therefore, we hypothesize that Adrecizumab induces a shift of ADM from another compartment (most likely the interstitium) into the circulation (**Figure 2C**).Whether bio-ADM concentrations after Adrecizumab administration also increase in patients with septic shock remains to be investigated, although based upon preclinical available data, this is to be expected.

Another effect of Adrecizumab is the prolongation of the half-life of endogenous ADM: Spiking of Adrecizumab to ADM-containing serum samples *in vitro* strongly increased the half-life of ADM in a dose-dependent manner (**Figure 3**, based on unpublished data). A plausible mechanistic explanation for this observation is that binding of Adrecizumab to the N-terminus of ADM reduces accessibility for proteolytic decay, which is known to occur at the N-terminus of ADM[31].

Because Adrecizumab is only a weak inhibitor of ADM signaling, the net effect of Adrecizumab administration is that ADM activity on endothelial cells is not reduced, but rather increased. This allows for ADM levels in the circulation to exert the aforementioned beneficial effects on the endothelium (reduction of capillary leakage), whereas ADMs detrimental effects in the interstitium (vasodilation of VSMCs) may concurrently be reduced. Overall, we hypothesize that as a result of the weak inhibitory effects of the antibody, the net effects of ADM on endothelial cells are augmented by Adrecizumab, resulting in stabilization of vascular integrity (**Figure 2C**). This proposed mechanism of action also explains why the antibody directed against the C-terminus, that resulted in complete inhibition of ADM activity, did not improve sepsis outcome in preclinical studies[75].

Adrecizumab may have additional benefits over ADM for the treatment of sepsis. As alluded to before, ADM has a short half-life, therefore it needs to be infused continuously. Adrecizumab has a half-life of approximately 15 days, allowing administration of a single dose. Moreover, Adrecizumab does not have the adhesive properties that ADM has. Naturally, as our proposed mechanism of action is hypothetical, future experimental studies should focus on downstream signaling targets of ADM-pathways and measuring tissue and/or interstitial ADM concentrations.

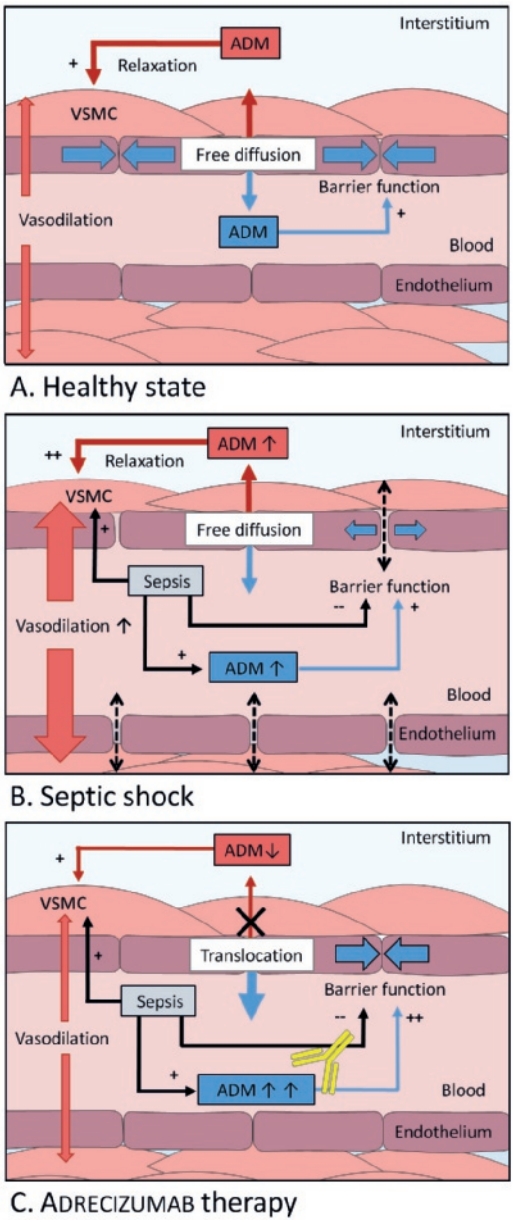

Figure 2. Hypothesized mechanism of action of Adrecizumab. **(A)** In the homeostatic 'normal' situation, Adrenomedullin (ADM) is able to diffuse freely over the vascular barrier and exert effects on endothelial cells and vascular smooth muscle cells (VSMC). **(B)** Sepsis-induced vasodilation and impaired vascular barrier function contribute to the development of shock. Circulating ADM levels are increased, but not to an extent that sufficiently counteracts impaired barrier function. Moreover, increased ADM levels lead to excessive vasodilation. **(C)** Following administration of Adrecizumab (yellow), ADM concentrations in the circulation increase rapidly, because of reduced proteolytic degradation and the inability of Adrecizumab-ADM complexes to cross the endothelial barrier. As there is no increased ADM synthesis, we hypothesize that ADM translocates from the interstitium to the circulation. As ADM that is bound to Adrecizumab remains functional, there is a net enhanced beneficial effect of ADM on endothelial cells (stabilization of the barrier function), whereas ADMs detrimental effects on VSMCs (vasodilation) are reduced due to a lower concentrations in the interstitial compartment.

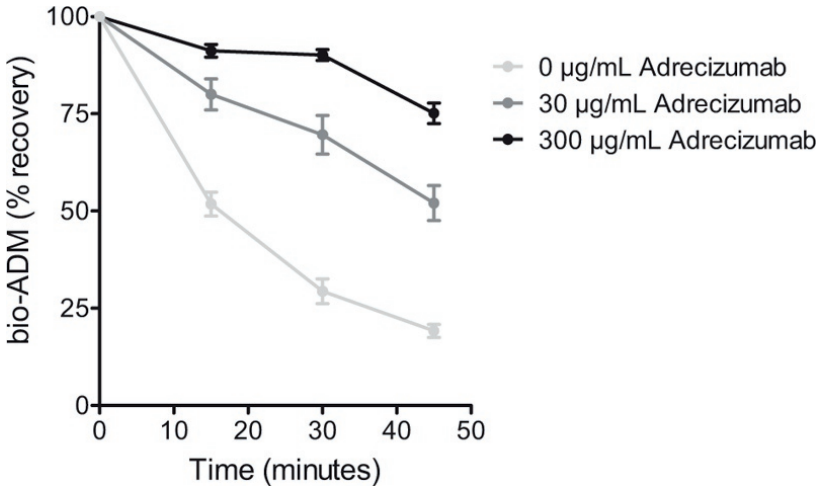

Figure 3. Serum samples (three pools from healthy donors) were spiked with a high concentration of synthetic ADM (640 pg/mL) in the absence or presence of Adrecizumab (0, 30, 300 µg/mL). The ADM concentration used was similar as in very severe septic shock, and the 30 µg/mL Adrecizumab concentrations was in the range measured in plasma shortly after in vivo administration of 2 mg/kg Adrecizumab, a dose used in preclinical experiments[77]. After incubation at 22°C for increasing time intervals, aliquots of the samples were measured in the sphingotest® bio-ADM assay. In the absence of Adrecizumab, bio-ADM concentrations decreased by approximately 80% after 45 minutes of incubation, whereas they decreased by approximately 50% and 25% in the presence of Adrecizumab at 30 and 300 µg/mL, respectively (both $p<0.001$).

Safety of Adrecizumab

The safety and tolerability of Adrecizumab were extensively investigated in preclinical animal studies. Toxicology and safety was studied in three different animal species. In short, Adrecizumab was well tolerated and safe up to the highest dosages tested (400 mg/kg), which are about hundredfold over the preclinically efficacious and intended clinical therapeutic dose (unpublished data). Moreover, application of up to 50 mg/kg Adrecizumab did not influence the blood pressure in telemetered beagle dogs (unpublished data). Recently a human phase I study was successfully completed (ClinicalTrials.gov identifier NCT02991508), and excellent safety results were observed[80].

Possible limitations

In the preclinical studies discussed before, Adrecizumab was administered prior to, or immediately after endotoxin administration or the cecal ligation

and puncture procedure. This approach is common for proof of principle studies, but represents a relevant limitation, as pretreatment of septic patients is unattainable in clinical practice. Moreover, previous work has convincingly shown that Adrecizumab administration results in a rapid and potent increase of circulating ADM, both in healthy animals and humans as well as in inflamed and/or septic animals[77,78,80]. Because ADMs vasodilatory effects are mediated not only through direct effects on VSMCs (which are presumably reduced by Adrecizumab [**Figure 2C**]), but also through indirect effects on endothelial cells, the increase in ADM levels could theoretically also cause unwanted vasodilation and subsequent aggravation of hypotension in septic shock patients. However, the relative importance of the indirect endothelial cell pathway compared to the direct effects on VSMCs in the vasodilatory effects of ADM remains to be determined. Nevertheless, no hypotensive effects were observed after Adrecizumab administration in telemetered healthy beagle dogs (unpublished data) and healthy human volunteers[80]. Furthermore, in the aforementioned preclinical study in CLP-induced sepsis in mice[76], lower norepinephrine infusion rates were observed in the treatment group, which strongly suggests that Adrecizumab did not exert vasodilatory effects. Finally, it is unclear whether the long half-life of Adrecizumab is a benefit or a limitation of the compound. While it enables single dosing treatment, increasing the feasibility of the therapy, this also may represent a limitation if unwanted effects of the drug may occur. Taken together, although there are currently no indications of hypotensive effects of Adrecizumab, the compound has not been investigated in septic patients yet, so no definitive conclusions can be drawn on the safety profile in patients. Moreover, it is imperative that future research also encompasses administration of ADM after the inflammatory and/or infectious insult to increase clinical relevance.

Other endothelial barrier stabilizing agents

Current guidelines do not recommend any adjunctive treatment for sepsis, except for hydrocortisone in patients with septic shock who respond poorly to fluid resuscitation and vasopressor therapy[82]. Although several adjunctive interventions (mainly anti-inflammatory agents) have been investigated in septic patients, up to now no clinical trials have been performed that have investigated endothelial barrier-stabilizing agents. This is slowly changing: Currently, the short-acting selective vasopressor 1a receptor agonist selepressin is under investigation in a study aiming to include 1800 patients with septic shock (ClinicalTrials.gov NCT02508649). Preclinical data suggest that

this selective V1-agonist[83] can improve vascular leakage by ligation of V1a receptors. In two studies employing ovine models of septic shock induced by burn injury combined with Pseudomonas aeruginosa pneumonia or CLP, selepressin decreased fluid accumulation compared with vasopressin and norepinephrine, indicating decreased vascular leakage[84,85]. Also, in a recent phase IIa study, selepressin was an effective substitute for norepinephrine, and in one dosage group an improved fluid balance was observed[86]. Another important regulator and potential target for endothelial barrier-stabilizing therapy is the angiopoietin-Tie-2 receptor pathway[87,88]. Several studies have demonstrated that Tie-2 agonists such as angiopoietin-1 (ang1) and modifications/alterations of ang1 which increase the half-life and binding efficacy, can prevent capillary leakage in animal models of septic shock[89-91], whereas targeted angiopoietin-2 inhibition also appears to hold potential[92]. Another potential treatment target with respect to endothelial barrier function is vascular endothelial growth factor (VEGF)[93], a potent inducer of endothelial barrier hyperpermeability[94], although the body of evidence is small and negative studies have also been reported[95]. It remains to be determined whether these strategies are truly effective in septic patients and whether one agent holds advantage over another.

Conclusion

ADM freely diffuses over the endothelial cell layer. In the blood compartment ADM improves endothelial barrier function, while in the interstitium it causes vascular smooth muscle relaxation. During sepsis, higher ADM concentrations are found which may aggravate vasodilation. As the non-neutralizing anti-adrenomedullin antibody Adrecizumab does not cross the endothelium, we hypothesize that ADM distribution is shifted towards the circulation where it is still functional after binding to Adrecizumab. Adrecizumab was shown to enhance endothelial barrier function in experimental models of systemic inflammation and sepsis, in the absence of untoward (vasodilatory) effects. Furthermore, administration of the antibody improved clinical outcomes, while safety and tolerability studies so far showed no side effects. These results pave the way for further clinical studies with Adrecizumab, which will have to reveal whether these effects are also present in patients with sepsis.

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Chapter 4

The mechanism of action of the adrenomedullin-binding antibody Adrecizumab

Christopher Geven and Peter Pickkers

Critical Care 2018; 22(1): 159

Letter to editor

With interest we read the review by Levy et al.[1] that provides a thorough overview of current and future therapies aiming to treat vasoplegia, an ubiquitous phenomenon in shock. In their work, the authors dedicated a section to the novel adrenomedullin (ADM)-binding antibody adrecizumab, which is mentioned as an ADM-blocking compound in the section title. Recently published studies demonstrate that blocking of ADM does not accurately describe Adrecizumab's mechanism of action, though. In contrast to what is often intuitively assumed, not all antibodies completely inhibit the activity of their targets. The extent of signaling inhibition can vary greatly, depending on the epitope to which the antibodies bind and other factors, such as antibody concentrations.

In contrast to C-terminus binding anti-ADM antibodies which completely inhibit ADM signaling, antibodies against the N-terminus of ADM, including the humanized monoclonal antibody adrecizumab, only marginally inhibit ADM activity, despite their high affinity and even when applied in vast molar excess over ADM[2].

Interestingly, animal and human data reveal a strong, dose-dependent increase of plasma ADM concentrations upon adrecizumab infusion[3,4], which cannot be explained by increased production of ADM. A mechanistic explanation for this occurrence was recently proposed[5]. Excess antibody that remains in the circulation is thought to drain ADM from the interstitium into the circulation, since ADM is small enough to cross the endothelial barrier, whereas the antibody is not. While adrecizumab only partially inhibits ADM signaling, the strong concentration increase of ADM (complexed with adrecizumab) in the circulation is thought to result in an overall 'net' increase of ADM activity on endothelial cells, augmenting endothelial barrier stabilizing effects, while decreased concentrations of ADM in the interstitium reduce vasodilatory effects on vascular smooth muscle cells. The combination of attenuation of both endothelial leakage and vasodilation likely represents the therapeutic potential of adrecizumab. This hypothesis fits well with previous studies that showed beneficial effects of ADM agonists in animal models of shock, while complete inhibition of ADM did not improve outcome.

As is mentioned by the authors, a proof-of-concept and dose-finding phase II study with adrecizumab is currently ongoing in patients with septic shock and elevated levels of ADM (ClinicalTrials.gov identifier NCT03085758). This trial incorporates a number of innovative features such as a novel composite efficacy endpoint and biomarker-guided patient selection (enrolment based on bio-ADM level), making it one of the first personalized treatment trials in sepsis.

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Chapter 5

Adrenomedullin in heart failure: pathophysiology and therapeutic application

Adriaan A. Voors, Daan Kremer, Christopher Geven, Jozine ter Maaten, Joachim Struck, Andreas Bergmann, Peter Pickkers, Marco Metra, Alexandre Mebazaa, Hans-Dirk Düngen and Javid Butler

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Abstract

Adrenomedullin (ADM) is a peptide hormone first discovered in 1993 in pheochromocytoma. It is synthesized by endothelial and vascular smooth muscle cells and diffuses freely between blood and interstitium. Excretion of ADM is stimulated by volume overload to maintain endothelial barrier function. Disruption of the ADM system therefore results in vascular leakage and systemic and pulmonary oedema. In addition, ADM inhibits the renin–angiotensin–aldosterone system. ADM is strongly elevated in patients with sepsis and in patients with acute heart failure. Since hallmarks of both conditions are vascular leakage and tissue oedema, we hypothesize that ADM plays a compensatory role and may exert protective properties against fluid overload and tissue congestion. Recently, a new immunoassay that specifically measures the biologically active ADM (bio-ADM) has been developed, and might become a biomarker for tissue congestion. As a consequence, measurement of bio-ADM could potentially be used to guide diuretic therapy in patients with heart failure. In addition, ADM might be used to guide treatment of (pulmonary) oedema or even become a target for therapy. Adrecizumab is a humanized, monoclonal, non-neutralizing ADM-binding antibody with a half-life of 15 days. Adrecizumab binds at the N-terminal epitope of ADM, leaving the C-terminal side intact to bind to its receptor. Due to its high molecular weight, the antibody adrecizumab cannot cross the endothelial barrier and consequently remains in the circulation. The observation that adrecizumab increases plasma concentrations of ADM indicates that ADM-binding by adrecizumab is able to drain ADM from the interstitium into the circulation. We therefore hypothesize that administration of adrecizumab improves vascular integrity, leading to improvement of tissue congestion and thereby may improve clinical outcomes in patients with acute decompensated heart failure. A phase II study with adrecizumab in patients with sepsis is ongoing and a phase II study on the effects of adrecizumab in patients with acute decompensated heart failure with elevated ADM is currently in preparation.

Introduction

Hospitalizations for heart failure are a major burden for patients, relatives, health care providers and society. Despite improvements in therapy for patients with heart failure with a reduced ejection fraction, we have not been able to reduce the risk of heart failure readmissions after a hospitalization for worsening heart failure. Approximately 35–50% of heart failure patients are rehospitalized within 6 months of discharge, making heart failure the most frequent diagnosis for 30-day readmissions and incurring billions in costs[1-3]. Therefore, preventing hospital (re-)admissions is recognized as a major unmet need in the treatment of heart failure.

Risk prediction models have become reasonably accurate for predicting (cardiovascular) mortality, but models to predict hospital (re-)admissions perform much worse[4]. Given its poor predictive value, identifying patients for clinical trials who are at high risk of hospital (re-)admission is difficult. Therefore, there is a need for markers to better identify patients who are at high risk for heart failure (re-)hospitalization. Preferably, these markers should reflect a process that can be pathophysiologically linked to worsening heart failure.

Several studies have consistently shown that the main reason for (re-) hospitalization for worsening heart failure is related to dyspnoea or breathlessness, mainly caused by pulmonary congestion[5-9.] The great majority of patients are treated with loop diuretics to relieve congestion, but a large number of patients are discharged without losing body weight and with persistent signs of congestion[7, 9-11]. This is particularly true when patients are discharged early and in patients without weight loss despite loop diuretic therapy. Consequently, studies have shown higher risks of hospital readmission in patients with a shorter duration of hospitalization, with a poor diuretic response, and with residual signs of congestion at discharge. Therefore, markers that reflect residual congestion might be useful to identify patients that are both epidemiologically and pathophysiologically at higher risk of hospital (re-)admission. Currently, clinical signs at physical examination such as oedema, rales and jugular venous pressure are the mainstay for assessment of congestion. However, this examination is often not performed in clinical practice, is very dependent on the clinician's skills[12], has a low interrater reliability and specificity[13-15] and is subjective due to the lack of standardized metrics or (de)congestion scores[16]. In view of the high incidence

of rehospitalization, clinical assessment of congestion is clearly insufficient. What clinicians therefore need is an easy-to-use surrogate for the assessment of a patient's (de)congestion status that may facilitate clinical decision making. The use of biomarkers for this purpose is attractive, since they are objective, easily available and have a known sensitivity and specificity. While by many heart failure clinicians natriuretic peptides are regarded as the main biomarker for congestion, recent studies show that there are several limitations in the use of natriuretic peptides as markers of congestion[17]. Since natriuretic peptides reflect stretch and pressure of the heart, they mainly reflect intravascular volume overload, but do not reflect tissue and interstitial fluid. Therefore, better and more specific markers for tissue congestions are needed.

Adrenomedullin: an introduction

Adrenomedullin (ADM) is a peptide hormone discovered in 1993 by Kitamura et al.[18.] ADM is a 52 amino acid peptide, containing a ring structure and a C-terminal amide, both of which are essential for binding to ADM receptors. The ADM gene is located on chromosome 11 and consists of four exons[19]. One of the key determinants for biological activity of ADM is the group of receptor activity-modifying proteins (RAMPs). The combination of RAMP 2 or 3 with the calcitonin receptor-like receptor (CRLR) confers specificity of the receptor for ADM, and thus both RAMPs and CRLR are key in ADM expression[20-25]. While ADM was first discovered in pheochromocytoma originating from the adrenal medulla (hence the name 'adrenomedullin'), further investigation showed that it was synthesized by many other tissues/cells, especially endothelial and vascular smooth muscle cells, and due to its small size (6 kDa) diffuses freely between blood and interstitium[26,27]. By proteolytic fragmentation of the pro-hormone (pro-ADM), a glycine-extended, inactive ADM is formed, which subsequently is enzymatically converted from ADM-glycine to the biologically active ADM-amide. ADM receptors and binding sites are widespread in the body, but cardiovascular and lung tissues have highest density of these binding sites[28]. The *in vivo* half-life of ADM is approximately 22 min[29]. ADM is thought to mainly be metabolized by neutral endopeptidase, also known as neprilysin[30] – a molecule that clinicians might recognize from the recent beneficial findings with sacubitril/valsartan as a novel treatment for patients with heart failure[31]. Sacubitril is a neprilysin inhibitor, and thus is supposed to inhibit breakdown of ADM and several other peptide hormones. An additional mechanism by which ADM is cleared is through binding with its receptors and subsequent internalization and degradation[32,33].

Vascular effects of adrenomedullin

The most recognized function of ADM is vasodilatation in both vascular resistance and capacitance vessels. ADM lowers blood pressure, yet increases blood flow[18,34]. Even low doses induce vasodilatation, indicating that the plasma levels of ADM under conditions such as heart failure are in the range that directly affect vascular tone[34].

Beside vasodilation, ADM seems to play an important role in preservation of endothelial integrity. ADM expression can be induced by various stimuli, one of them being volume overload, and increased plasma ADM reflects excessive fluid volume[35]. This is most likely the consequence of a counteracting response, as the ADM-induced stabilization of endothelial barrier function is thought to limit tissue fluid overload. Indeed, disruption of the ADM system results in vascular leakage and systemic and pulmonary oedema[36-38]. Also the role of the ADM–RAMP 2 system has been investigated. Mice lacking the gene encoding for RAMP 2 showed enhanced vascular permeability and systemic oedema[36]. Similarly, mice with a conditional knock-out of ADM in endothelial cells revealed increased vascular permeability in comparison with wild-type littermates[39].

Further support for the effects of ADM in maintaining vascular integrity comes from experimental studies showing that experimental overexpression of ADM inhibits systemic and pulmonary vascular leakage in animals[40-42]. For example, in a rat model of Staphylococcus aureus-toxin induced systemic inflammation, accompanied by extensive vascular leakage, ADM infusion protected endothelial barrier function via cyclic adenosine monophosphate (cAMP) elevation[40]. Also, ADM dose-dependently reduced experimentally induced endothelial hyperpermeability of cultured human umbilical vein endothelial cell and porcine pulmonary artery endothelial cell monolayers[41]. Suppression of ADM contributes to vascular leakage and altered epithelial repair during asthma[42]. In two animal models, intranasal ADM completely attenuated the acute-induced airway hyper-responsiveness and mucosal plasma leakage[42,43]. ADM acts on several pathways in order to stabilize the endothelial barrier, including the cAMP/protein kinase A (PKA) pathway that inhibits RhoA/ROCK and reduces subsequent myosin light chain kinase-induced actomyosin contraction (the 'pulling forces' exerted on endothelial cell junctions), as well as the cAMP/PKA and possibly the PI3K/Akt pathway to promote production of (protective) cortical actin and stabilization of the

VE-cadherin/β-catenin complex (part of adherens junctions)[44]. Finally, ADM inhibits the renin–angiotensin–aldosterone system[45]. Although ADM increases plasma renin activity, it induces reductions in the aldosterone/plasma renin activity ratio and attenuates angiotensin II-induced aldosterone secretion. In addition, ADM is upregulated by angiotensin II, and protects against cardiac hypertrophy and renal damage induced by angiotensin II. Altogether, it is suggested that ADM acts as a functional antagonist to angiotensin II, hereby inhibiting aldosterone secretion and thus compensating for renin–angiotensin–aldosterone system escalation.

In summary, vasodilatation and maintaining vascular integrity are the two most important functions of ADM. Importantly, the effects of ADM depend on its location. ADM is present both intravascular and in the interstitium. Its mode of action intravascular as opposed to the interstitium is depicted in **Figure 1**. Intravascular ADM is thought to improve vascular integrity and decrease vascular permeability through its effects on endothelial cells. Interstitial ADM however is thought to cause vasodilatation by acting on vascular smooth muscle cells, in an endothelium-independent mechanism. Note that endothelial-dependent pathways have also been described, although it remains unknown to what extent each pathway is involved *in vivo* in humans[46-48].

Adrenomedullin is elevated in heart failure and related to congestion and clinical outcome in heart failure

In healthy humans, ADM circulates in the plasma in low concentrations. In 1995, it was first reported that ADM levels were elevated in heart failure[49]. Plasma ADM concentration was 13 pg/mL in healthy subjects and 3 to 4 times higher in patients with chronic heart failure[49]. The observation that ADM levels decreased after treatment with diuretics and digitalis led to the assumption that 'volume expansion and an activated sympathetic nervous system may be associated with this increase and that plasma ADM levels change in response to the pathophysiologic changes of heart failure[50]. After this, many studies have shown elevated levels of ADM in patients with heart failure. In addition, several studies found a strong association between higher levels of ADM and adverse clinical outcome[51,52]. The majority of these studies used a stable part of the ADM precursor peptide, mid-regional pro-ADM (MR-proADM)[53]. The drawback to this assay, however, is that it measures a stable fragment of a non-functional ADM pro-peptide, and therefore does

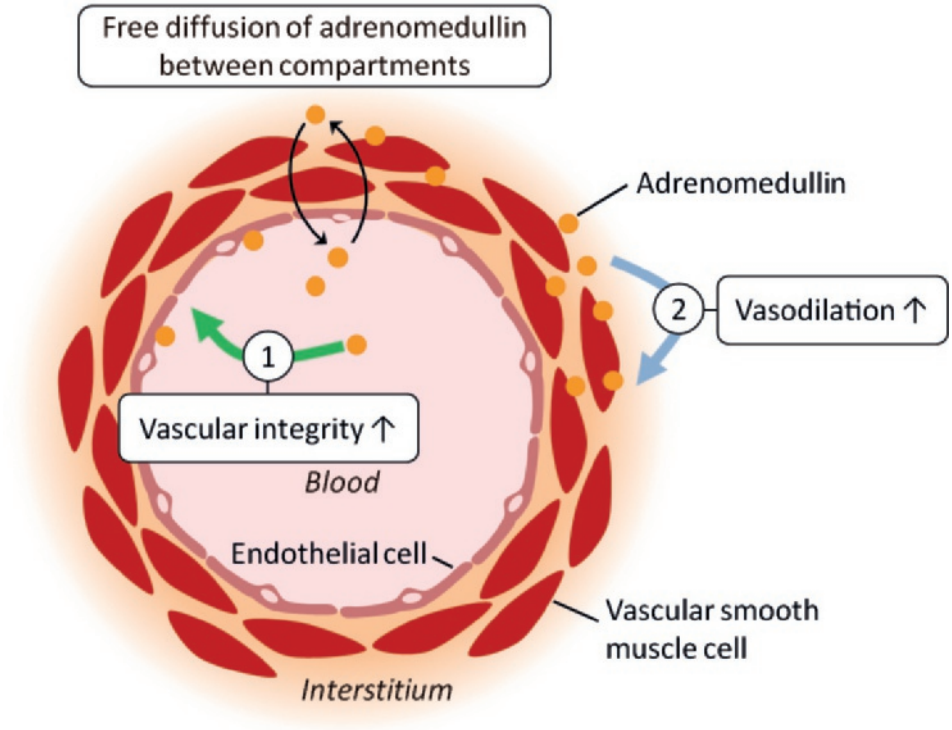

Figure 1. Simplistic representation of the mode of action of intravascular vs. interstitial adrenomedullin. **1.** Adrenomedullin present within the blood vessels improved vascular integrity, thereby putatively reducing vascular permeability. **2.** Adrenomedullin present in the interstitium acts on the vascular smooth muscle cells and causes dilatation of the vascular resistance and capacitance vessels.

not distinguish between the biologically active amidated ADM and the non-functional ADM variant containing a glycine-extended C-terminal residue. Recently, a new immunoassay that specifically measures biologically active ADM (bio-ADM) has been developed[53]. Plasma bio-ADM was measured in 246 patients admitted at the emergency department with suspicion of acute heart failure[54]. Plasma bio-ADM concentrations were higher among patients who experienced a cardiovascular event (median 80.5 pg/mL; interquartile range [IQR] 53.7–151.5 pg/mL) compared with those who did not (median 54.4 pg/mL; IQR 43.4–78.4 pg/mL) ($P < 0.01$). After adjusting for the other biomarkers, plasma bio-ADM remained a strong predictor of a cardiovascular event[54]. Another study showed that bio-ADM was a marker of impaired haemodynamics, organ dysfunction, and poor prognosis in patients with cardiogenic shock[55]. We recently studied the clinical correlation

and prognostic value of serial measurements of plasma bio-ADM levels in 1562 patients admitted for acute decompensated heart failure[56]. We showed that plasma bio-ADM had the strongest association with clinically assessed congestion during hospital admission for acute decompensated heart failure. Moreover, bio-ADM was a better predictor of residual congestion than any other individual baseline variable. In patients with clinical signs of residual congestion 7 days after hospital admission, bio-ADM levels were high at baseline and remained high throughout the first week of hospitalization. This in contrast to brain natriuretic peptide (BNP) levels, which decreased in all patients irrespective of the presence and degree of residual congestion. Finally, plasma bio-ADM concentrations, both at baseline and at day 7, provided significant added predictive value for 60-day heart failure rehospitalization, even after adjustment for a pre-defined 11-item rehospitalization risk model, residual congestion by day 7 and BNP at day 7.

Rationale for bio-adrenomedullin as a biomarker for tissue congestion

Adrenomedullin is a vasoactive peptide that is increased in patients that are volume overloaded. Main functions of ADM are vasodilatation and to maintain vascular integrity and decrease vascular leakage. Elevated levels are found in heart failure, but ADM is particularly elevated in patients with septic shock. A common factor between both diseases is vascular leakage and organ hypoperfusion. In heart failure, higher levels of ADM are associated with more severe heart failure, and are the strongest predictor of (residual) congestion in patients with acute decompensated heart failure. Increased ADM concentrations have been associated with impaired clinical outcome in several studies of patients with heart failure. In a recent study, higher levels of bio-ADM were independently associated with a higher risk of hospital readmissions[56]. We therefore propose the following concept, as depicted in **Figure 2**. We propose that higher bio-ADM levels reflect residual tissue congestion, and residual tissue congestion is related to worse outcome after discharge, and a higher likelihood for frequent hospital readmissions in particular. Therefore, such a measurement might guide physicians to treat certain patients more intensively, and this might also facilitate discharge decisions.

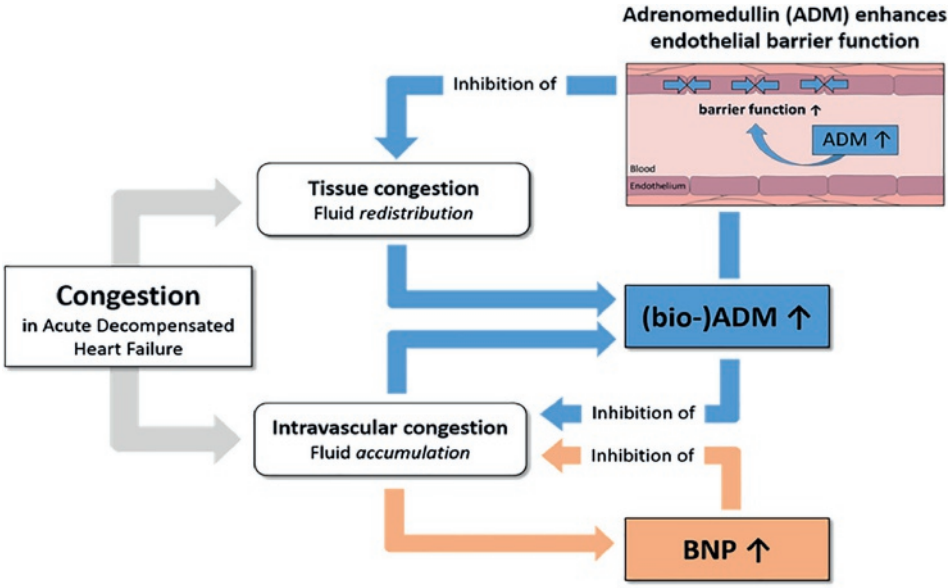

Figure 2. Bio-adrenomedullin (ADM) as a marker and inhibitor of tissue congestion. Brain natriuretic peptide (BNP) as a marker and inhibitor of intravascular congestion.

Adrenomedullin as a target for therapy

Several preclinical (**Table 1**)[57-66] and small clinical (**Table 2**)[67-70] studies have established the effects of exogenous administration of ADM in heart failure. Briefly, these effects included a reduction in myocardial infarct size, cardiac myocyte apoptosis, left ventricular remodelling (in animals) and aldosterone levels (animals and humans), while haemodynamics (in both humans and animals) and survival (in animals) were improved. In a rat coronary ligation model, ADM administration during the early period of a myocardial infarction improved survival and ameliorated progression of left ventricular remodelling and heart failure[62]. Similar results were found in another study where chronic administration of ADM attenuated transition from left ventricular hypertrophy to heart failure in rats[60]. In a case series of seven acute heart failure patients with dyspnoea and pulmonary congestion, the effects of long-term intravenous administration of ADM in acute decompensated heart failure were studied. ADM infusion significantly reduced mean arterial pressure, pulmonary arterial pressure and systemic and pulmonary vascular resistance without changing heart rate, and increased cardiac output for most time-points compared with those at baseline[69]. In another small study of seven chronic heart failure patients and seven healthy subjects, ADM

significantly decreased mean arterial pressure and increased heart rate in the healthy volunteers[68]. In patients with heart failure, ADM also decreased mean arterial pressure and increased heart rate, but to a much lesser degree. ADM markedly increased cardiac index while decreasing pulmonary capillary wedge pressure[68].

Table 1. Overview of preclinical studies investigating ADM in different models related to heart failure

Author (year)	Intervention	Animal model	Effects
Nakamura (2002)[57]	ADM infusion for 4 weeks via osmotic pump versus saline	Left coronary ligation-induced myocardial infarction in rats	↓ Heart weight/body weight ↓ Myocyte size ↓ Collagen volume fraction of non-infarct LV area, without affecting infarct size ↓ LV end-diastolic pressure
Okumura (2003)[58]	ADM infusion for 60 minutes after coronary ligation	Ischemia-reperfusion (30 min of left coronary artery ischemia) in rats	After 24 hours: ↓ LV end-diastolic pressure and ↓ cardiac myocyte apoptosis After 4 weeks: ↓ Myocardial fibrosis ↓ Myocardial infarct size
Niu (2003)[59]	Heterozygous ADM(+/-) knock-out mice compared to wild-type	Stress-induced cardiac hypertrophy by angiotensin II infusion	Knock-out resulted in: ↑ Cardiac hypertrophy (heart weight/body weight and LV thickness) ↑ Renal dysfunction
Nishikimi (2003)[60]	ADM infusion over 7 weeks using a micro-osmotic pump, compared with placebo and diuretic treatment groups	Heart failure model of Dahl salt-sensitive rats	ADM infusion: ↓ LV end-diastolic pressure, ↓ RV systolic pressure, ↓ RA pressure ↓ LV weight/body weight ↓ Renin-aldosterone & ANP ↑ Cardiac output & systemic vascular resistance ↑ LV end-systolic elastance ↑ Survival compared to diuretic and placebo
Okumura (2004)[61]	ADM infusion, or ADM + wortmannin, or placebo, for 60 minutes after coronary ligation	Ischemia-reperfusion (30 min of left coronary artery ischemia) in rats	↓ Infarct size ↓ LV end-diastolic pressure ↓ Myocardial apoptotic death Pretreatment with wortmannin abolished beneficial effects of ADM, indicating involvement of the PI3K/Akt dependent pathway

Taken together, these data consistently show that ADM induces beneficial haemodynamic, hormonal and myocardial changes in both experimental models and patients with heart failure. These effects are likely related to the vasodilatory properties of intravascular ADM, although other pathways may also be involved. Many vasodilators have been studied in acute and chronic heart failure with mixed results. Therefore, it would be more interesting to be able to stimulate the effects of ADM on endothelial permeability, and to prevent decreases in blood pressure, since this might be deleterious in patients with worsening heart failure.

Table 1. Continued

Author (year)	Intervention	Animal model	Effects
Nakamura (2004)[62]	I.p. ADM or placebo over 7 days, immediately after induction of myocardial infarction	Left coronary ligation-induced myocardial infarction in rats. Observed over 9 weeks	At 9-weeks: ↑ Survival, ↓ heart/lung weight ↓ Oxidative stress and ACE transcription ↓ LV end-diastolic pressure ↓ Collagen volume fraction of the non-infarcted LV. No effect on infarct size During 7-days of infusion: No effects on urinary output or hemodynamic parameters
Niu (2004)[63]	Heterozygous ADM(+/-) knock-out mice compared to wild-type	Stress-induced cardiac hypertrophy by aortic constriction or angiotensin II infusion	More pronounced in ADM knock-out mice: ↑ Heart weight/bodyweight ratio, ↑ LV wall thickness, ↑ perivascular fibrosis, ↑ Expression of ACE, angiotensinogen, collagen type 1, BNP and c-fos ↑ Renal damage with glomerular sclerosis Involvement of a PKA/PKC pathway
Looi (2006)[64]	ADM bolus	Left coronary ligation induced myocardial infarction in anesthetized rats	↓ Ventricular arrhythmias Involvement of NO mechanism
Yoshizawa (2016)[65]	Subcutaneous infusion of ADM using an osmotic minipump for 3 or 7 days	Doxorubicin-induced cardiac damage in mice	↑ 14 day survival ↓ LDH levels ↓ DOX-induced cardiac tissue damage, mitochondrial abnormalities and cell death
Li (2018)[66]	Myocardial transplantation of mesenchymal stem cells overexpressing ADM (ADM-MSC) compared to GFP-MSCs	Isoproterenol-induced global heart failure	↑ Cardiac function ↓ Cardiac fibrosis

Abbreviations: ACE, angiontensin-converting enzyme; ADM, adrenomedulline; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; GFP, green fluorescent protein; i.p., intraperitoneal; LV, left ventricular; MSC, mesenchymal stem cell, MSC; PKA, protein kinase A; PKC, protein kinase C; RA, right atrial; RV, right ventricular.

Table 2. Overview of studies investigating ADM in human patients with forms of heart failure

Author (year)	Intervention	Condition	Effects
Nakamura (1997)[67]	FBF and SBF test with intra-arterial ADM	Healthy subjects (n=10) Patients with CHF (n=10)	↑ FBF and ↑ SBF Effects partially NO-mediated Impaired FBF and SBF responses in CHF group
Nagaya (2000)[68]	I.v. infusion of ADM (n=7) or placebo (n=6), 90 min total	Patients with precapillary pulmonary hypertension (n=13 total)	↑ Cardiac index (44%) ↓ Pulmonary arterial pressure (32%) ↓ MAP (9 mmHg), ↑ HR ↓ Plasma aldosterone (but not renin) No effect ANP/BNP
Nishikimi (2009)[69]	I.v. infusion of ADM + hANP for 12 h, followed by 12 h of hANP	Acute heart failure patients with dyspnoe and pulmonary congestion (n=7)	ADM + hANP: ↓ MAP, PAP, systemic and pulmonary vascular resistance ↑ Cardiac output, urine volume and urine sodium excretion ↓ Aldosterone, BNP, free-radical metabolites
Kataoka (2010)[70]	I.v. infusion of ADM for 12 h No placebo arm	Patients with acute myocardial infarction before undergoing PCI (n=10)	During infusion, two patients showed unstable hemodynamics MRI 3 vs. 1 month after PCI: ↑ Wall motion index, ↓ infarct size

Abbreviations: ADM, adrenomedullin; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CHF, chronic heart failure; FBF, forearm blood flow; hANP, human atrial natriuretic peptide; HR, heart rate; MAP, mean arterial pressure; MRI, magnetic resonance imaging; NO, nitric oxide; PAP, pulmonary artery pressure; PCI, percutaneous coronary intervention; SBF, skin blood flow.

Adrecizumab for the treatment of heart failure and sepsis

Adrecizumab is a humanized, monoclonal, non-neutralizing antibody against the N-terminus of ADM. It has a half-life of 15 days when administered by a single intravenous infusion. The mode of action of adrecizumab is presented in **Figure 3**. Administration of adrecizumab leads to a dose-dependent increase in plasma ADM bound to the administered antibody. The increase occurs within a few minutes and is not caused by induction of de-novo synthesis, because concentrations of MR-proADM are not increased (an inactive peptide fragment originating from the same precursor as ADM, which is synthesized in a 1:1 ratio). It is hypothesized that translocation of pre-existing ADM accounts for the observed increase in circulating ADM[71]. Briefly, circulating adrecizumab cannot leave the blood compartment due to its high molecular weight (160 kDa), whereas ADM (with a much lower molecular weight of 6 kDa) can freely cross the endothelial barrier between the interstitium and the circulation[55]. Binding of ADM by adrecizumab (present in the circulation

in a large excess over ADM) prevents ADM from leaving the blood vessel, effectively 'trapping' ADM in the circulation. In addition, adrecizumab may translocate ADM from the interstitium into the circulation. Because adrecizumab is a non-neutralizing antibody, the net effect is a significant increase of functional plasma ADM which leads to – as we hypothesize – the restoration of vascular integrity (endothelial effect) and less vasodilatation (vascular smooth muscle cell effect due to decreased concentrations of ADM in the interstitium). Further, it is thought that adrecizumab prevents ADM from being degraded by proteases and prolongs half-life of ADM[71].

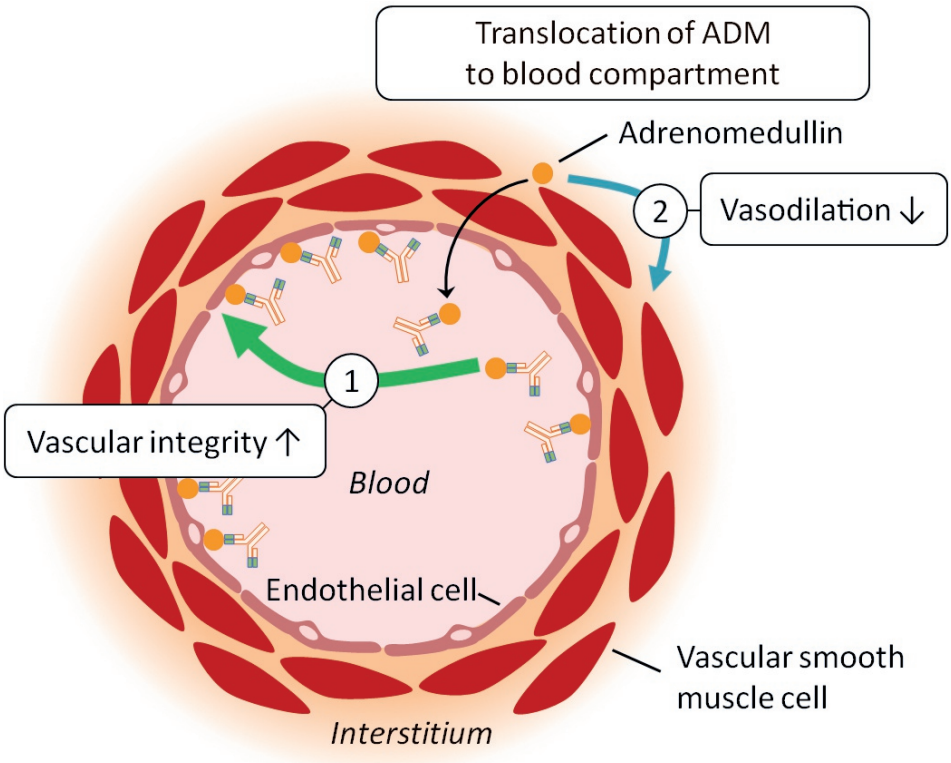

Figure 3. Mode of action of Adrecizumab. Administration of adrecizumab leads to a dose-dependent increase of plasma adrenomedullin (ADM) bound to the administered antibody. Circulating adrecizumab cannot leave the blood compartment due to its high molecular weight (160 kDa), whereas ADM (with a much lower molecular weight of 6 kDa) can freely cross the endothelial barrier between the interstitium and the circulation. Binding of ADM by adrecizumab (present in the circulation in a large excess over ADM) prevents ADM from leaving the blood vessel, effectively 'trapping' ADM in the circulation.

In animal models of systemic inflammation and septic shock, adrecizumab improved haemodynamics, renal function, systemic inflammation and reduced inducible nitric oxide synthase expression[71]. Currently, adrecizumab is being evaluated in a phase II trial in human septic shock (http://clinicaltrials.gov identifier: NCT03085758). There were no safety concerns observed in pre-clinical studies, as well as in two phase I studies in which 0.5, 2 and 8 mg/kg were administered to healthy volunteers[71]. Importantly, even though adrecizumab induced great increases in circulating levels of ADM, this did not cause hypotension. In a phase Ib study, where healthy subjects received an infusion of bacterial lipopolysaccharide to induce a systemic inflammatory response, administration of adrecizumab significantly reduced the perception of illness/sickness, as assessed by clinical scores[72].

A phase II proof of concept study in patients with worsening heart failure and elevated bio-ADM levels after initial stabilization is currently being considered. Patients with elevated bio-ADM have (residual) congestion and are at high risk for clinical events, and a heart failure readmission in particular. Adrecizumab is expected to increase intravascular ADM and decrease interstitial ADM, since adrecizumab may translocate ADM from the interstitium into the circulation. The hypothesis of this study is that by improving vascular integrity, it is expected that adrecizumab will decrease tissue congestion and thereby improve dyspnoea, potentially reducing heart failure readmissions. However, it should be noted that long-term effects of ADM on development and/or progression of heart failure have never been clinically investigated. In addition, adrecizumab itself has not been investigated in pre-clinical models of heart failure, but only in septic shock and systemic inflammation. Tissue congestion was never an outcome parameter in pre-clinical studies with ADM.

Conclusions

Adrenomedullin is an endogenous hormone that is released as a counteracting response to volume overload. Levels of ADM are clearly increased in patients with heart failure, and higher levels are related to more advanced heart failure and worse outcomes. Since elevation of ADM is a feedback response to volume overload to maintain vascular integrity and decrease vascular leakage of fluid from the the vasculature to the tissues, measurement of ADM might reflect tissue and pulmonary oedema. Such a measurement might guide physicians to more intensively treat patients with heart failure hospitalization and facilitate discharge decisions. In addition, ADM might become a target of therapy in heart failure. The mode of action of adrecizumab, a humanized monoclonal antibody that binds but does not significantly inhibit ADM, might be of particular interest, since it is assumed to translocate ADM from the interstitium into the vasculature to improve vascular integrity and prevent vascular leakage. A clinical study with adrecizumab is currently being conducted in patients with sepsis and a study in hospitalized heart failure patients is currently being prepared.

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Chapter 6

Experimental human endotoxemia as a model of systemic inflammation

Christopher Geven, Dirk van Lier, Guus Leijte and Peter Pickkers

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Abstract

Systemic inflammation plays a pivotal role in a multitude of conditions, including sepsis, trauma, major surgery and burns. However, comprehensive analysis of the pathophysiology underlying this systemic inflammatory response is greatly complicated by variations in the immune response observed in critically ill patients, which is a result of inter-individual differences in comorbidity, comedication, source of infection, causative pathogen, and onset of the inflammatory response. During experimental human endotoxemia, human subjects are challenged with purified endotoxin (lipopolysaccharide) intravenously which induces a short-lived, well-tolerated and controlled systemic inflammatory response, similar to that observed during sepsis. The human endotoxemia model can be conducted in a highly standardized and reproducible manner, using a carefully selected homogenous study population. As such, the experimental human endotoxemia model does not share the aforementioned clinical limitations and enables us to investigate both the mechanisms of systemic inflammation, as well as to evaluate novel (pharmacological) interventions in humans in vivo. The present review provides a detailed overview of the various designs, organ-specific changes, and strengths and limitations of the experimental human endotoxemia model, with the main focus on its use as a translational model for sepsis research.

Introduction

Systemic inflammation plays a pivotal role in a multitude of conditions, including sepsis, trauma, major surgery, and burns[1]. For many years, efforts have been undertaken to unravel the complex etiology of the systemic inflammatory response. Unfortunately, comprehensive analysis of the pathophysiology underlying this response is greatly complicated by variation in the immune responses observed in critically ill patients, which are a result of inter-individual differences in comorbidity, comedication, source of infection, causative pathogen, and onset of the inflammatory response. The heterogeneity between patients impedes evaluation of pathophysiological mechanisms and hampers accurate comparison of (pharmacological) interventions. As a consequence, large numbers of patients need to be included in clinical trials to demonstrate intervention efficacy. And strikingly, even when these numbers were met, many of the positive results found in preclinical (animal) studies of systemic inflammation could not be reproduced in expensive (phase III) clinical trials[2,3]. Therefore, an intermediate step is highly warranted to improve translation of preclinical animal data to sepsis patients, which will likely prevent more disappointing results, and may allocate resources more efficiently.

The experimental human endotoxemia model can be used to overcome the aforementioned constraints of translating preclinical results into clinical practice. During experimental human endotoxemia, volunteers are challenged with purified endotoxin (lipopolysaccharide [LPS]) derived from the Gram-negative bacterium Escherichia coli. An intravenous challenge with LPS induces a short-lived, well-tolerated, and controlled systemic inflammatory response, mimicking the initial inflammatory response observed in septic patients[4]. Importantly, these challenges can be conducted in a highly standardized and reproducible manner in a carefully selected (homogeneous) study population. To this end, experimental human endotoxemia does not share the aforementioned limitations of clinical trials[4,5] and therefore is an example of translational research that facilitates us to investigate the mechanisms of systemic inflammation and to evaluate novel (pharmacological) interventions in humans *in vivo*[5].

The aim of the present review is to provide a detailed overview of the various designs, organ-specific changes, and strengths and limitations of the experimental human endotoxemia model, with the main focus on its use as a translational model for sepsis research.

The human endotoxemia model

For over a century, the experimental human endotoxemia model has been successfully applied using LPS in different types, dosages, and forms of administration[1,4,5]. The models used are safe, well-tolerated, and bear no known long-term health risks to the participants. The most frequently described method is by intravenous administration of LPS, although other forms have also been described, e.g., intrabronchial administration[6]. The cornerstone of the endotoxemia model is the interaction of LPS with the Toll Like receptor (TLR)-4, an interaction which also constitutes the first step in the inflammatory cascade in for example Gram-negative sepsis[4,7]. Intravenous LPS administration elicits a transient and controlled systemic inflammatory response, clinically characterized by an increase in core temperature of approximately 1.5–2 °C, flu-like symptoms (such as headache, chills, fatigue, myalgia, backache, and nausea) during 2–4 h, as well as hemodynamic alterations (tachycardia, tachypnea, and decrease in blood pressure).

Experimental setup

After approval of the local ethics committee and written informed consent, all subjects are thoroughly screened prior to inclusion (using medical history, physical examination, laboratory tests, and a 12-leads electrocardiogram). The procedure and general requirements for conducting a LPS challenge are displayed in **Figure 1**.

Dose regimens

Currently, the only commercially available LPS for use in humans is the GMP-grade E. Coli Type O113 (Lot no. 94332) produced by List Biological Laboratories in Campbell, USA. The use of different doses have been well described in literature, varying from extremely low dosages of 0.1–0.3 ng/kg (only causing minor cytokine peaks with the absence of clinical symptoms), to a maximum used dose of 4 ng/kg (resulting in a profound systemic inflammatory response accompanied by the aforementioned clinical symptoms)[4,8,9]. The magnitude of the inflammatory response after LPS is highly dose-dependent and therefore, higher dosage regimens are mainly used as an in vivo translational model for sepsis, while the lower dosage ranges are increasingly applied to model a state of low grade inflammation, which is observed in e.g. diabetes and metabolic syndrome[9,10]. While most studies use the traditional single bolus administration of LPS, a recent study proposed a novel method consisting of a bolus administration of 1 ng/kg followed by

a continuous administration of 1 ng/kg/h for 3 h[1]. This continuous model resulted in a more prolonged inflammatory response compared to bolus LPS administration, exemplified by higher plasma cytokine concentrations and circulating leukocytes, and an increased duration of fever and clinical symptoms, while no safety issues were reported. Therefore, the continuous model may better resemble the ongoing inflammation seen during sepsis, and may also provide a larger (and clinically more relevant) time window to examine the effects of immunomodulatory interventions.

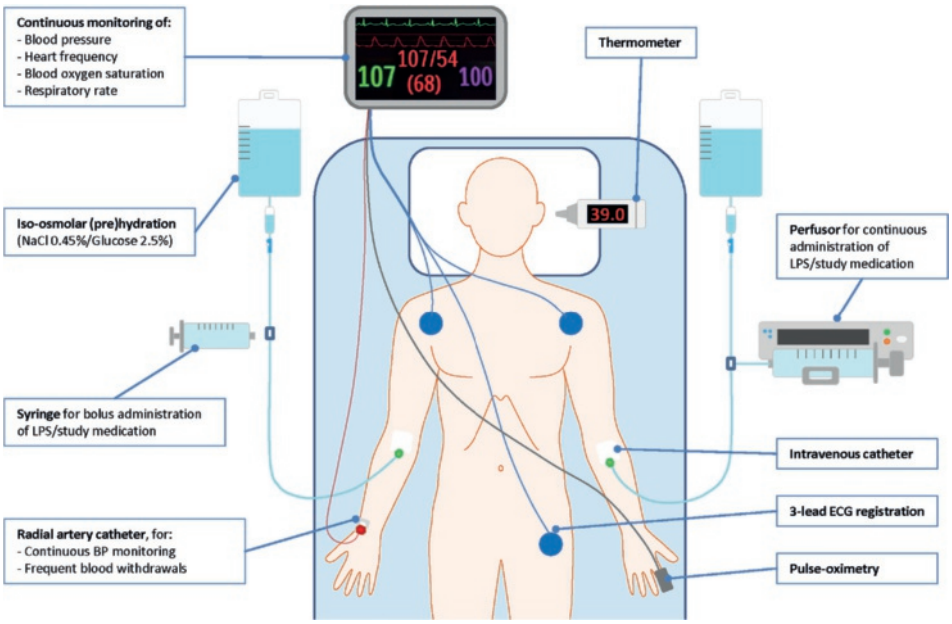

Figure 1. Overview of the experimental setup used to conduct experimental human endotoxemia. On the experiment day, subjects are hospitalized and receive one or two (18 gauge) venous catheters to accommodate infusion of LPS, fluids, and study medication. Subsequently, an arterial catheter is placed, preferably in the radial artery of the nondominant arm, for frequent blood withdrawals and blood pressure monitoring. Vital signs are continuously monitored during the experiment using 3-lead electrocardiography, peripheral pulse-oximetry, and the intra-arterial blood pressure signal. Body temperature (determined using an ear thermometer) and severity of symptoms are determined every half hour. Approximately 1 h prior to LPS administration, subjects are prehydrated with 1.5 L NaCl 0.45%/Glucose 2.5% solution, as this is known to reduce the risk of a vasovagal reaction following endotoxemia[54]. Hereafter, fluid administration is continued at a rate of 150 mL/h until the end of the experiment. Arterial blood is drawn at baseline and serially during the endotoxemia to determine leukocyte differentiation and cytokine concentration over time. Furthermore, depending on the research questions proposed, the evaluation of numerous parameters and/or (pharmacological) interventions can be added to the protocol. *Abbreviations: LPS, lipopolysaccharide; BP, blood pressure; ECG, electrocardiography; NaCl, sodium chloride.*

Repeated experimental human endotoxemia

The immune response in septic patients is highly variable and can comprise both a hyperinflammatory as well as an immune-suppressed phenotype. The latter state, also known as sepsis-induced immunoparalysis, is increasingly recognized as the overriding immune dysfunction in sepsis, rendering patients vulnerable to secondary infections and impairing sepsis outcome. However, the identification of immunoparalyzed patients remains difficult, this is why few clinical studies have investigated immunostimulatory therapies. Interestingly, LPS administration results in a phenomenon called 'endotoxin tolerance', which is characterized by severely attenuated cytokine responses and less pronounced changes in clinical symptoms upon a second challenge, compared to the first LPS challenge[11,12]. Endotoxin tolerance bears many hallmarks of (sepsis-induced) immunoparalysis, such as a decreased ex vivo cytokine production of LPS-stimulated monocytes and a decreased HLA-DR expression on monocytes[11]. To this end, the repeated human endotoxemia model can be used to model sepsis-induced immunoparalysis, allowing us to investigate immunostimulatory therapies in humans in vivo, without the aforementioned clinical constraints[11]. Please note that the similarities between endotoxin tolerance and immunoparalysis are mainly observed in the innate immune response, as LPS administration does not strongly affect the adaptive immune response.

Experimental human endotoxemia-induced organ-specific effects

The intravenous administration of LPS, especially within the high dose ranges (2–4 ng/kg), exerts many organ-specific effects. Since the model is conducted in a highly standardized and reproducible manner, it offers an unique opportunity to investigate the pathophysiology underlying these changes and to evaluate new (organ-specific) interventions[9]. In the following section, we complement previous reviews on experimental endotoxemia[4,5,8] and focus on the effects of experimental human endotoxemia on important organ systems, and recite the most important interventions concerning each organ system. An overview of the LPS-induced organ-specific effects is displayed in **Figure 2**.

The immune system

Upon administration, LPS monomers bind to TLR-4-MD2 receptor-domains expressed on antigen presenting cells, which leads to an activated TLR-4 dimeric receptor complex. Mediated by adaptor protein MYD88,

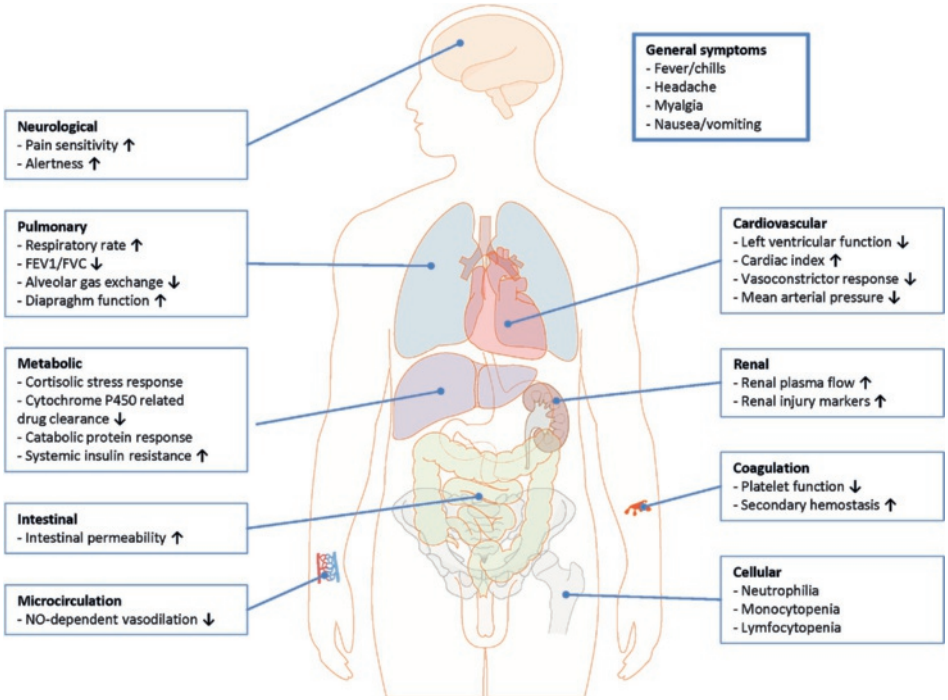

Figure 2. Summary of experimental human endotoxemia-induced organ-specific effects. Overview of the organ-specific effects resulting from intravenous administration of LPS within the high dose ranges (2-4 ng/kg). *Abbreviations: FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity.*

several intracellular protein-complex-pathways are subsequently activated, resulting in the activation of the transcription factor NF-kB. Binding of NF-kB to its target genes triggers the synthesis of RNA-sequences which are translated to various pro- and anti-inflammatory cytokines. Plasma cytokine levels upon LPS administration have a distinct, dose-dependent and highly reproducible time course[1,7,8]. Tumor necrosis factor (TNF)-α is the first to peak (90–120 min) and is believed to be the primary mediator leading to the LPS-induced systemic inflammatory cascade. TNF-α is closely followed by peak concentrations of IL-1β, IL-6, but also of the anti-inflammatory cytokine IL-10, all of which have maximum plasma concentrations at approximately 3 h following LPS administration. Within 6–8 h after the LPS challenge, all plasma cytokines levels return to baseline values[1].

The inflammatory cytokine responses observed during experimental human endotoxemia, as well as a multitude of interventions targeted against it have

been extensively studied. A study investigating gender-based differences during experimental endotoxemia, found significantly higher peak TNF-α concentrations in females, while no difference in the anti-inflammatory IL-10 cytokine response was observed[13]. Arguably, this more pronounced pro-inflammatory response in females could partially explain both the lower incidence, and the better outcome of sepsis in female patients. Prednisolone, a well established anti-inflammatory therapeutic, was found to reduce the release of pro-inflammatory cytokines TNF-α and IL-6 during endotoxemia in a dose dependent manner, while it enhanced the release of the anti-inflammatory cytokine IL-10[14]. These findings might explain the high susceptibility to infection in patients often observed after prolonged use of corticosteroids.

Using repeated human endotoxemia as a model for sepsis-induced immunoparalysis, several immunostimulatory agents have been investigated for their effect on preventing or restoring the suppressed immune function. Of interest, while ex vivo endotoxin tolerance (ex vivo re-stimulation of leukocytes with LPS) resolves quickly, in vivo endotoxin tolerance (in vivo re-challenge with LPS) persists for at least 2 weeks[15]. Based on the poor correlation between ex vivo and in vivo responses, clearly ex vivo endotoxin tolerance kinetics do not accurately reflect the in vivo innate immune response, further emphasizing the need for an in vivo translational model of endotoxin tolerance. Indeed, in a recent double-blind randomized controlled trial it was shown that treatment with IFN-γ during repeated experimental endotoxemia could partially reverse endotoxin tolerance[11]. These in vivo results further support the hypothesis that immunostimulatory agents could be a promising treatment option to reverse sepsis-induced immunoparalysis.

Hematology

Sequestration of immune cells results in neutrophilia, and a mono- and lymphocytopenia, with lowest counts at 2 and 4 h following intravenous administration of LPS, respectively[1]. Interestingly, a recent study using deuterium labeling to monitor immune cells, reported that the repopulation of monocytes is achieved by the early release of classical monocytes from the bone marrow, and not by return of monocytes from the marginating pool (monocytes that do not circulate as they adhere to the endothelium)[16]. Another study investigating the function of different neutrophil subsets during experimental endotoxemia found a down-regulation of neutrophil receptors

necessary for chemotaxis, microbial recognition and killing[17]. Moreover, a moderately decreased interaction with opsonised Staphylococcus epidermidis bacteria was found[17]. Intriguingly, these suppressed neutrophil phenotypes were also associated with a marked upregulation of the capacity to produce reactive oxygen species (ROS), which are essential for neutrophil bactericidal activity. These apparently contradictory findings may be explained by priming and activation of already circulating neutrophils by LPS leading to the enhanced ROS production on one hand, while LPS also induces the release of refractory, immature neutrophils with lower antimicrobial functionality from the bone marrow.

The cardiovascular system

Endotoxin-induced systemic inflammation has profound effects on the heart and vascular system. In the first hours following endotoxin administration, a hyperdynamic cardiovascular state is present, characterized by decreased systemic vascular resistance, lower arterial blood pressure and blunted responsiveness to sympathetic vasoconstrictors, as well as increased cardiac output and heart rate[1,18-20]. Myocardial contractility parameters (including left ventricular ejection fraction) typically show a biphasic response, with an initial increase, but later decrease (even though the hyperdynamic state persists for 6–12 h)[18,19,21]. Experimental human endotoxemia therefore represents (at least to a certain extent) cardiovascular manifestations of sepsis, and can be used to study the cardiovascular effects of interventions. For example, an antagonist against the toxic lipid A component of LPS (E5531) inhibited the early hyperdynamic cardiovascular response, as well as the late myocardial depression[21] and recombinant human activated protein C (APC) prevented the decrease in mean arterial blood pressure (MAP) during human endotoxemia[22].

The microcirculation and vascular endothelial permeability

Extensive microcirculatory alterations are present during sepsis, resulting in a mismatch of oxygen supply and demand due to shunting[23]. In addition, tissue edema develops due to vascular hyperpermeability combined with the need for fluid resuscitation. This contributes to development of for instance acute respiratory distress syndrome (ARDS) and acute kidney injury (AKI)[24]. It is well known that LPS administration can cause profound vascular leakage in animals[25], and indeed, plasma obtained during human endotoxemia causes vascular leakage in vitro[26]. In contrast, an increased capillary leakage could

not be detected in humans in vivo, indicating that the experimental human endotoxemia model is not suitable to study microvascular permeability[27]. Prolonged duration and increased severity of the inflammatory response observed in animal models of endotoxemia, as a consequence of higher dose administration of LPS (approx. 1000 times higher) likely explain this discrepancy. Note that there is one report that describes the case of a laboratory worker self-administering an extremely high dose of Salmonella endotoxin. This resulted in severe shock with non-cardiogenic pulmonary edema and multi-organ failure, indeed similar to septic shock[28]. Even though hyperpermeability is not present in the experimental human endotoxemia model, several other manifestations of endothelial and vascular dysfunction can be observed. These include reduced tissue oxygenation (using near-infrared spectroscopy in combination with upper-arm ischemia), reduced blood flow in medium and large microvessels (using sidestream dark-field imaging) and an attenuation of acetylcholine-induced vasodilation through forearm blood flow measurements[29]. In addition, plasma levels of different endothelial dysfunction/adhesion markers are increased, such as endocan, E-selectin, P-selectin, VCAM-1 and ICAM-1, although in contrast to sepsis, angiopoeitin-1, Tie2, thrombomodulin and VE-cadherin are not increased[30-35].

The kidneys

The development of AKI is common in sepsis and associated with increased mortality[36]. AKI is thought to arise through a complex mechanism of immune-mediated microvascular and tubular dysfunction[37]. Similar to sepsis, several urinary tubular damage markers are also increased following experimental human endotoxemia. For example, glutathione-S-transferase-A1 (GSTA1-1), a marker of proximal tubule damage, was increased in urine and correlated with increased nitric-oxide (NO) metabolite excretion[38]. Interestingly, co-administration of the inducible nitric oxide synthase (iNOS) inhibitor aminoguanidine during human endotoxemia reduced upregulation of iNOS mRNA, urinary NO metabolites and urinary GSTA1-1, suggesting a role of iNOS and NO in renal proximal tubule damage[38]. More recently, significant increases of urinary B2MG and KIM-1, markers indicating (subtle) proximal tubular damage were reported. In contrast, many other markers, among which serum creatinine, were not affected[35]. Also note that these increases of urinary B2MG and KIM-1 were 3–4 fold less pronounced as compared to septic AKI. Therefore, one may conclude that there is only subtle kidney injury during human endotoxemia, making its clinical relevance as a model for septic AKI questionable.

Pulmonary system

In addition to intravenous administration, LPS has also been administered intrapulmonary to induce a local inflammatory response in the bronchoalveolar space[6], which bears features of acute lung injury and/or pneumonia[6]. In this pulmonary LPS model, intravenous co-administration of activated protein C (APC) resulted in both anti-coagulatory and anti-inflammatory effects in the bronchoalveolar space[6,39], whereas intrapulmonary APC administration led to a pro-coagulatory and pro-inflammatory phenotype[40]. Moreover, changes in respiratory capacity and pulmonary gas exchange were recently investigated following both systemic and pulmonary endotoxin administration[41]. Both models decreased forced expiratory volume (FEV1) and forced vital capacity (FVC), although this effect was more pronounced after intravenous LPS administration. Furthermore, the alveolar-arterial oxygen gradient increased after intravenous, but not after intrapulmonary LPS administration[41].

Respiratory muscles

Systemic inflammation is thought to contribute to respiratory muscle weakness in mechanically ventilated patients, which may lead to weaning problems[42]. A recent study investigated whether LPS affects diaphragm function, and if it could act as a model of respiratory muscle weakness. In contrast to animal studies and findings in critically ill patients, in vivo diaphragm contractility was not impaired, but rather augmented during experimental human endotoxemia, possibly related to the release of stress hormones[43].

Coagulation

Experimental human endotoxemia induces platelet activation and consumption, prolonged APTT and an increased INR[44,45]. In addition, increased fibrinolysis and fibrinogen consumption is reported, which is followed by downregulation of fibrinolysis. Primary hemostasis is reduced, whereas secondary hemostasis is enhanced[45]. Different interventions that act on coagulation and/or fibrinolysis have been examined using the human endotoxemia model. Colistin, which electrostatically interacts with LPS[46], blunted endothelial activation and the fibrinolytic response, while activation of the coagulation system was not severely affected. This suggests that colistin may exert other effects besides its antimicrobial activity in patients with Gram-negative sepsis[46]. In a study with APC (also mentioned earlier), no effects were observed on fibrinolysis, coagulation or inflammation during human endotoxemia[22]. Interestingly, the human endotoxemia model is not necessarily limited to application in healthy subjects. Factor V Leiden (FVL)

mutations (causing hypercoagulability) were initially thought to worsen clinical outcome in patients with disseminated intravascular coagulation in sepsis, although clinical trials and animal data later indicated the opposite. Therefore, a study was conducted to explore the mechanism of action of the FVL mutation during human endotoxemia[47]. Interestingly, males with a heterozygous FVL mutation showed an enhanced fibrinolytic response to endotoxin, possibly due to higher levels of soluble fibrin acting as cofactor in tissue plasminogen-induced plasminogen activation. This may therefore result in better clearance of fibrin deposits, a reduction of fibrinogen levels and generation of 'anticoagulant' fibrinogen degradation products.

The gastrointestinal tract

During sepsis, the permeability of the intestinal barrier increases, allowing translocation of bacteria and luminal contents (e.g. pancreatic enzymes)[36]. Thirty years ago, the increased intestinal permeability following endotoxin administration was first reported, when a study found urinary secretion of the (normally) non-metabolizible sugars lactulose and mannitol after oral ingestion[48]. Results were confirmed in another study using polyethylene glycol, indicating that inflammation-induced paracellular permeability and not ischemia-mediated enterocyte damage was responsible for the increased intestinal permeability[49]. Interestingly, increased permeability seems to be limited to the small intestine[50].

The gut microbiota is known to have an important interaction with gut-barrier function and modulation of the immune system during sepsis[51]. In addition, sepsis and/or antibiotics influence gut-microbiota composition. Nevertheless, antibiotic-induced alterations in microbiota composition did not influence inflammation during experimental human endotoxemia, even though gut microbiota diversity was decreased by broad-spectrum antibiotics[52]. Moreover, no endotoxemia-induced increase in liver damage marker GSTA1 was observed, indicating that the human endotoxemia model is probably too mild to cause inflammation-induced liver injury[53].

Limitations of the human endotoxemia model

To date, the experimental human endotoxemia is the only in vivo model of systemic inflammation. However, several important factors may limit its clinical relevance and extrapolation to clinical research in sepsis. A summary of the main differences between experimental human endotoxemia and sepsis is

displayed in **Table 1**, a summary of the main similarities is displayed in **Table 2**. The differences listed in **Table 1**, as well as those mentioned earlier for the different organ systems, underline that while some aspects of experimental endotoxemia are highly comparable to sepsis, others are markedly different (e.g. capillary leakage, liver injury, and an adaptive immune response). Considering the complex pathophysiology of systemic inflammation, experimental endotoxemia should therefore never be envisioned as an alternative or direct comparison to sepsis, but rather function as an important bridge between preclinical (laboratory and animal) studies on one side, and the indispensable clinical research in patients on the other side.

Table 1. Differences between experimental human endotoxemia and sepsis.

	Experimental human endotoxemia	Sepsis
Subject characteristics	- Homogeneous population - (Mostly) young adults - (Mostly) healthy - (Mostly) males	- Heterogeneous population - Average age >50 years - Comorbidities - Comedication
Inflammatory cascade	- Highly standardized (known time of onset) - LPS-induced - TLR-4 mediated - Predominantly innate immune response	- Unknown time of onset - Live pathogens - Multi-causative - Innate and adaptive immune response
Cytokines	- Short elevation of plasma cytokines (hours)	- Prolonged cytokine response (days-weeks)
Cardiovascular and clinical features	- Short 'hyperdynamic response' with limited decrease in blood pressure - No capillary leak/shock symptoms	- Sustained and profound hemodynamic compromise often leading to multi-organ failure - Capillary leak leading to substantial edema formation
Risks/outcome	- Transient flu-like symptoms - Well-tolerated - Safe - No long-term effects	- Up to 40% mortality - Significant (long-term) morbidity in survivors, often need for medical rehabilitation

Abbreviations: LPS, lipopolysaccharide; TLR, Toll-like receptor.

Table 2. Similarities between experimental human endotoxemia and sepsis

	Similarities
General symptoms	- Fever response with characteristic 'chills and shivering' - Headache, myalgia, nausea and vomiting
Inflammatory cascade	- Innate immunity mediated cytokine release
Cerebral	- Decreased pain threshold
Cellular response	- Marked leukocytosis - Induction of immune suppression upon secondary challenge - Induction of nitric oxide release - Induction of hepcidin-mediated anemia
Cardiovascular	- Reduced left ventricular contractility - Hyperdynamic circulation with elevated cardiac index - Induction of endothelial dysfunction
Pulmonary	- Tachypnea - Reduced alveolar gas exchange
Renal	- Increased urinary excretion of tubular injury markers
Coagulation	- Reduced platelet count and function - Increased secondary hemostasis
Metabolic	- Catecholamine and cortisolic stress response - Reduced insulin sensitivity - Catabolic protein response
Gastrointestinal	- Increased gut permeability

Future improvements on the model

Research performed during the past decade has also focused on ways to further improve the comparability between experimental human endotoxemia and sepsis. As mentioned earlier, in a recently introduced continuous model of (high dose) LPS administration, bolus administration was followed by continuous LPS infusion during three hours[1]. This leads to more sustained and higher plasma cytokine concentrations, more circulating leukocytes, and an increased duration of fever and clinical symptoms compared to LPS bolus injection models. The continuous LPS infusion model may therefore better resemble the state of ongoing inflammation as observed during sepsis. Future research should concentrate on further optimizing LPS dosage regimens, possibly through even longer durations of continuous LPS administration.

Conclusion

Experimental human endotoxemia is a translational model of systemic inflammation in humans in vivo and has been successfully performed in thousands of healthy volunteers. It has proven to be safe, well-tolerated and without any known long-term health risks for the participating subjects. Unlike the heterogeneous inflammatory response observed in sepsis patients, the inflammatory responses observed during experimental endotoxemia are mono-causative (TLR-4 driven), highly standardized, and reproducible, greatly facilitating the possibilities for between-subject and intervention-based comparisons. The overlap in inflammatory response, clinical parameters, and organ-specific changes emphasize that experimental human endotoxemia may serve as a model for sepsis. However, the model also has limitations, implying that direct comparisons between experimental endotoxemia and clinical causes of systemic inflammation should always be made with caution. Nevertheless, the multitude of studies performed on the observed cytokine responses, the different inflammation-induced organ dysfunctions, as well as therapeutic interventions directed against these have been indispensable in advancing our understanding of the complex pathophysiology of systemic inflammation and sepsis. Therefore, the experimental human endotoxemia model should be used to facilitate the translation of preclinical work to the sepsis population.

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Part II

Adrenomedullin as a biomarker in the critically ill

Chapter 7

Bioactive adrenomedullin, organ support therapies and survival in the critically ill: Results from the FROG-ICU study

Lea Lemasle, Alice Blet, Christopher Geven, Ményssa Cherifa, Benjamin Deniau, Alexa Hollinger, Marie-Céline Fournier, Xavier Monnet, Esabelle Rennuit, Michael Darmon, Pierre-François Laterre, Joachim Struck, Oliver Hartmann, Andreas Bergmann, Alexandre Mebazaa and Etienne Gayat

Submitted

Abstract

Objectives: Adrenomedullin (ADM) is a vascoactive peptide and elevated ADM levels have been detected in sepsis. We assessed the relationship between circulating ADM, the need for organ support and mortality in a general intensive care unit (ICU) population, using a novel assay that measures biologically active ADM (bio-ADM).

Design: Prospective multicenter observational cohort study.

Setting: Data from the FROG-ICU study (French and European Outcome reGistry in Intensive Care Units).

Patients: Consecutive patients admitted to intensive care with a requirement for invasive mechanical ventilation and/or vasoactive drug support for more than 24 h following ICU admission were included.

Interventions: Clinical and biological parameters were collected at baseline, including bio-ADM. Status of ICU survivors was assessed up until 1 year after discharge. The main outcome was the need for organ support, including renal replacement therapy (RRT) and/or for inotrope(s) and/or vasopressor(s). Secondary endpoints included the ICU length of stay (LOS) and the 28-day all-cause mortality.

Measurements and main results: Median plasma bio-ADM (n=2003) was 66.6 [34.6-136.4] pg/ml and the median SAPS-II score 49 [36-63]. RRT was needed in 23% and inotropes(s) and/or vasopressor(s) in 77% of studied patients. ICU LOS was 13 [7-21] days and mortality at 28 days was 22%. Elevated bio-ADM independently predicted 1) the need for organ support (OR [95% CI] 4.02 [3.08-5.25]) in ICU patients whether admitted for septic or non-septic causes and 2) the need for RRT (OR 4.89 [3.83- 6.28]), and for inotrope(s) and/or vasopressor(s) (OR 3.64 [2.84-4.69]), even in patients who were not on those supports at baseline. Elevated bio-ADM was also associated with a prolonged LOS (OR 1.85 [1.49-2.29]) and, after adjustment for SAPS-II, with mortality (OR 2.31 [1.83-2.92]).

Conclusions: Early measurement of bio-ADM is a strong predictor of the need of organ support and of short-term mortality in critically ill patients.

Introduction

Adrenomedullin (ADM) is a 52 amino-acid peptide hormone belonging to the calcitonin gene-related peptide family. ADM has strong vascular actions[1,2] and has previously been described as a 'double-edged sword'[3]: preclinical studies have shown that ADM administration causes vasodilation[4–6] and may lead to hypotension[7,8], whereas other studies have demonstrated beneficial effects on endothelial cells in the prevention of vascular leakage[9–11] and improved outcome in preclinical animal models of systemic inflammation and septic shock[12,13].

Previous studies have assessed ADM activity in sepsis patients by measuring levels of its inactive precursor MR-proADM[14], or by using assay of uncertain validity or with technical difficulties[15]. However, the measurement of the bioactive form of ADM (bio-ADM) may better reflect the biological relevance. Using this novel sandwich immunoassay, two studies in sepsis and septic shock patients demonstrated that bio-ADM correlated with disease severity, the need for organ support and mortality[15,16]. Additional measurements over the first week after sepsis onset allowed for a better prediction of long-term mortality[15]. Whether baseline bio-ADM is associated with short-term outcome, especially the need of organ-support, and long-term outcome, in all Intensive Care Unit (ICU) patients and in patients admitted for causes other than sepsis, remains unclear.

The present study is an ancillary study from the FROG-ICU (the French and European Outcome Registry in Intensive Care Units) study[17]. The FROG-ICU study was a prospective, observational, multicenter cohort study that included septic and non-septic ICU patients, as well as a novel assay specific for bio-ADM. The main objective of the FROG-ICU study was to identify clinical and biological determinants of death in the year following ICU discharge. Multivariable analysis identified age, comorbidity, red blood cell transfusion, ICU length of stay and abnormalities in common clinical factors at the time of ICU discharge as independent factors associated with 1-year mortality. Bio-ADM was independently associated with 1-year death and when mixed in a panel of elevated biomarkers of cardiac and vascular failure improved the risk of death. The original FROG-ICU study focused on the risk of 1-year mortality according to the number of cardiovascular biomarkers elevated at discharge from the ICU. This study focuses on the relationship between baseline circulating bio-ADM, organ support therapies and short-term mortality in the critical ill. This is the first and largest prospective cohort study so far to investigate prognostic value of bio-ADM in the general ICU population.

Materials and methods

Study design and patients

Our study uses the data of the FROG-ICU study (ClinicalTrials.gov NCT01367093)[17]. The FROG-ICU study was a prospective, observational, multicenter cohort study, designed to assess the incidence and to identify the risk factors of mortality during the year following discharge from the ICU. The study protocol was previously published[18]. The study was conducted in France and in Belgium in accordance with Good Clinical Practice, the Declaration of Helsinki (2013, Fortalenza), and approved by the ethical committee. The study involved medical, surgical or mixed ICUs in 14 hospitals. The study cohort included 2087 consecutive patients who were admitted to the ICU in any of the participating centers during the recruitment period when the following inclusion criteria were met: invasive mechanical ventilation support for at least 24 h and/or treatment with a positive inotropic agent for more than 24 h. Exclusion criteria were the following: less than 18 years old, severe head injury (initial Glasgow Coma Scale < 8) or brain death or a persistent vegetative state, pregnancy or breastfeeding, transplantation in the past 12 months, not expected to survive or to leave the hospital and/or no social security coverage[17].

Data collection and biological samples

In the FROG-ICU study, for each patient included, an electronic case report form was completed that documented relevant information about the ICU stay. At the time of inclusion, the following patient data was collected: demographics, data on past medical history, ICU admission diagnosis, hemodynamic and respiratory parameters, and severity of disease classification scores. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-coated vials at patient inclusion. Bio-ADM was measured batch-wise in blinded fashion in aliquots of plasma stored at –40 °C, using a recently developed immunoassay[16,19]. In short, the assay is a one-step sandwichcoated tube chemiluminescence immunoassay, based on Acridinium NHS-ester labeling for the detection of human ADM in unprocessed, neat plasma. Further details on the immunoassay can be found in the Supplemental Material. Among the 2087 patients included in the FROG-ICU cohort, bio-ADM concentration at baseline was available for 2003 patients; this is our studied population.

Outcome

The main outcome was the need for organ-support therapies during the ICU stay (i.e. renal replacement therapy and/or inotrope(s) and/or vasopressor(s)) and secondary endpoints were ICU length of stay (ICU LOS) and 28-day all-cause mortality.

Statistical methods

Results are presented as count and percentage for categorical variable or median and first to third quartile range for continuous variables. Comparison between groups was performed using chi-square tests or Wilcoxon tests as appropriate. We tested the normality of the biomarker bio-ADM with the Shapiro test (p-value < 0.001), and we dichotomized the variable according to previously defined cut-off value of 70 pg/mL[16]. First, we studied the requirement of organ support therapies by recording the need of renal replacement therapy (RRT) and/or inotrope(s) and/or vasopressor(s) treatment during ICU stay. As inotrope(s) and/or vasopressor(s) treatment is an inclusion criteria, we focused on the need of inotrope(s) and/or vasopressor(s) treatment in patients without inotrope(s) and/or vasopressor(s) treatment at admission. Association between baseline bio-ADM and secondary endpoints (length of stay in ICU and 28-day mortality) was also considered. Marginal associations of bio-ADM and SAPS-II with the outcome was studied by a Wilcoxon test and logistic regression. We estimated the association between bio-ADM and the outcome with adjustment for SAPS-II using logistic regression. The results of logistic regression analysis are presented as odds ratios (ORs) and 95% confidence interval (CI). The area under the receiver operating characteristic curve (AUC ROC) to predict the all cause 28-day mortality was determined. We used SAPS-II as a clinical score to predict 28-day mortality, as previously described[20]. We added the biomarker bio-ADM to this score and the change in AUC with and without the biomarker was compared using the DeLong test. We assessed the primary endpoint in several predefined subgroups. First we considered subgroups based on the four most prevalent causes of admission in ICU: patients with septic shock, neurologic disorder, out of hospital cardiac arrest and acute respiratory failure (ARF). Then, we considered subgroups based on severity (SAPS-II score) and comorbidities (Charlson score). Additionally, Kaplan-Meier survival plots were used to examine the relationship between baseline bio-ADM and 28 days ICU's survival time. In order to determine the relationship between levels of bio-ADM and outcome, we performed restricted cubic splines which explore

the linearity of the association between bio-ADM and outcome and ROC curves to determine the cut-off point; sensitivity analysis was performed with the latter cut-off point, SOFA (Sequential Organ Failure Assessment) score at admission and Charlson score. Incremental predictive value of bio-ADM was determined by elevated net reclassification improvement (NRI) and integrated discrimination improvement (IDI)[21]. A two-sided p-value of 0.05 was considered significant. All analyses were performed using R 3.2.3 statistical software (the R Foundation for Statistical Computing, Vienna, Austria). ROC curve analyses were performed using the package "pROC" and graphics were plotted using the package "ggplot2".

Results

Patient characteristics

Patients had a median age of 63 [51-74] years and were predominantly male (n = 1297, 65%). Other characteristics are presented in **Suppl. Table 1**. Main reasons for ICU admission were septic shock, ARF, acute neurological disorder, out-of-hospital cardiac arrest and cardiogenic shock. At admission, patients admitted in our ICUs were in severe conditions with median SAPS-II score at 49 [36-63] and median Charlson's score at 3 [1-5]. Mortality at 28 days was 22%.

Circulating levels of bio-ADM

The median level of plasma bio-ADM was 66.6 pg/ml [34.6-136.4] in our studied patients (n = 2003). Levels of circulating bio-ADM organized by causes of admission, Charlson and SAPS-II scores are presented in **Table 1**. Highest levels of circulating bio-ADM were observed in septic shock patients (99.7 pg/ml [62-194.4]) compared to the lowest, found in patients with neurological disorders (27.9 [18.4-48.2]) that had normal bio-ADM values (99^{th} percentile reported at 43 pg/mL[16]) (**Figure 1**). Furthermore, patients with the highest SAPS-II score (greater than the median of 49, n = 977) at admission had greater circulating baseline bio-ADM levels: 88.9 pg/ml [46.9-173.6] compared to the patients with SAPS-II score < 49 at admission (n = 1026, 49.6 pg/ml [28.2-101.8], p < 0.001). Plasma bio-ADM leves were increased in patients with chronic kidney disease and low eGFR at admission (**Suppl. Table 2**).

Table 1. Levels of baseline bio-ADM according to 28-day mortality in ICU.

	All	Alive	Dead	p-value
All patients (n=2003)	**67 [35-136]**	**56 [30-115]**	**105 [62-219]**	**<0.001**
Septic shock (n=465)	100 [62-194]	86 [54-165]	147 [87-284]	0.002
Neuro disorder (n=273)	28 [18-48]	26 [18-44]	47 [29-64]	<0.001
Out of hospital cardiac arrest (n=176)	52 [31-99]	43 [29-80]	75 [45-137]	<0.001
Cardiogenic shock (n=137)	72 [37-136]	64 [34-112]	104 [65-222]	<0.001
Acute respiratory failure (n=381)	53 [30-96]	47 [28-88]	78 [50-139]	<0.001
Charlson I (n=816)	46 [26-94]	42 [25-89]	86 [49-164]	<0.001
Charlson II (n=648)	72 [40-142]	64 [37-123]	105 [58-246]	<0.001
Charlson III (n=539)	98 [53-173]	87 [46-155]	121 [75-226]	<0.001
SAPS-II < 49 (n=1026)	59 [28-102]	46 [27-92]	94 [47-159]	<0.001
SAPS-II ≥ 49 (n=977)	89 [47-174]	76 [39-146]	114 [67-244]	<0.001

Abbreviations: SAPS-II score, Simplified Acute Physiology II Score

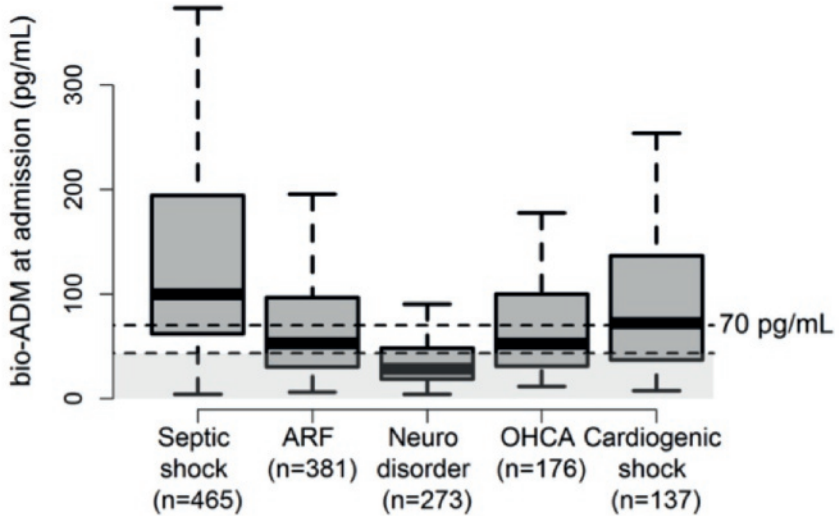

Figure 1. Bio-ADM levels according to the five most frequent causes of admission. Grey shadow area indicates normal range (< 43 pg/mL). The cut-off value of 70 pg/ml, used to define high versus low level of ADM, is highlighted by a dotted line. The dark band in each box represent the median value of the subgroup. The upper limit of the box represents the third quartile, and the lowest limit the first quartile. The dotted vertical line represents the 95% confident interval. *Abbreviations; ARF, acute respiratory failure; OHCA, out of hospital cardiac arrest.*

Association between baseline levels of bio-ADM and the need for organ-support therapies during ICU stay

Characteristics of studied patients (n = 2003) are presented in **Suppl. Table 1.** Among studied patients, 23% needed RRT and 77% inotrope(s) and/or vasopressor(s) during the ICU stay. **Suppl. Table 1** further describes patients characteristics based on baseline bio-ADM level. At admission, 564 (20%) patients were not treated with vasopressors and/or inotropes and none were on RRT.

Figure 2A shows that high levels of bio-ADM at baseline were significantly associated with the need for organ-support therapies (OR [95% CI] 4.02 [3.08-5.25]) in all ICU patients and in patients admitted for septic (OR [95% CI] 3.21 [1.22-8.43]) or non-septic cases (OR [95% CI] 3.57 [2.69-4.74]). High levels of bio-ADM were associated with the need of RRT during ICU stay in all studied ICU patients (adjusted OR [95% CI] 4.89 [3.83-6.28]) and also in patients with a good renal function at admission (OR [95% CI] 3.98 [2.21-7.18]). High levels of bio-ADM were also associated with the need for inotrope(s) and/or vasopressor(s) during ICU stay in all studied patients (OR [95% CI] 3.64 [2.84-4.69]), and in ICU patients without inotrope(s) and/or vasopressor(s) treatment at admission (OR [95% CI] 3.7 [2.36-5.83]) (**Figure 2A** and **Suppl. Table 3**). The latter subgroup had a bio-ADM level greater than bio-ADM of patients who never received inotrope(s) and/or vasopressor(s) during their ICU stay (**Suppl. Figure 1**).

Likewise, the association between plasma bio-ADM, renal dysfunction, need of vasopressors or 28-day mortality remain high even after adjustment for SOFA score alone or for SOFA and Charlson scores (**Suppl. Table 4**). Furthermore, incremental predictive value of bio-ADM was confirmed by elevated net reclassification improvement (NRI) and integrated discrimination improvement (IDI) NRI: 37.1% (95% CI: 27.1 – 47.1] and IDI: 0.021 (95% CI: 0.013 – 0.029] compared to SOFA for mortality and compared to SOFA for risk of RRT. Thus, with the bio-ADM value, 37% of patients are better classified.

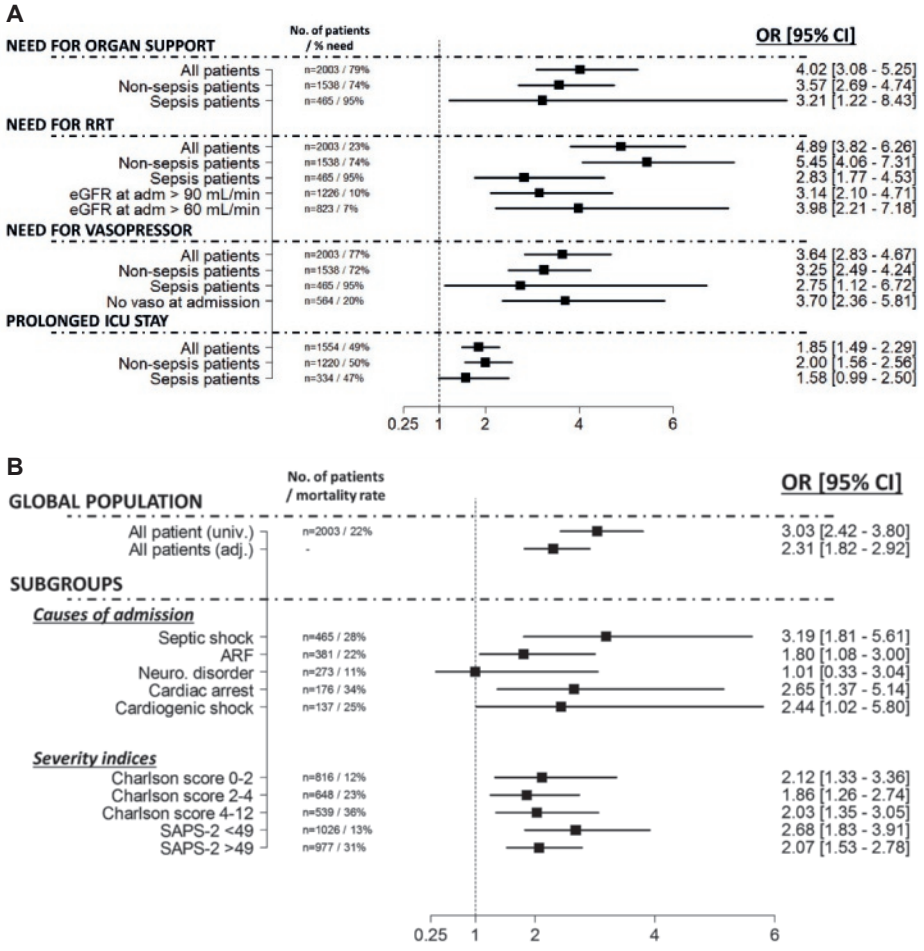

Figure 2. Relation between high baseline bio-ADM level (≥ 70 pg/mL) and the need for life-support therapies (**A**) and with all-cause 28-day mortality (**B**). High level of ADM is defined as ≥ 70 pg/mL. Prediction of organ support by bio-ADM was performed among patients without this organ dysfunction at admission. Need of organ support refers to any need at admission and/or during the ICU stay. Need of RRT was only seen during ICU stay. Need of vasopressor refers to any need at admission and/or during the ICU stay for all patients (non-septic and septic), and exclusively after admission for patients who were not treated with vasopressors at admission. *Abbreviations; ARF, acute respiratory failure; bio-ADM, bioactive adrenomedullin; SAPS-2, Simplified Acute Physiology Score II.*

Association between baseline levels of bio-ADM, lenght of stay in ICU and 28-day mortality

The median ICU length of stay was 13 [7-21] days. High baseline bio-ADM levels were associated with prolonged ICU stay in survivors (OR [95%] 1.85 [1.49-2.29]). Concerning survival during the first 28 days, 441 patients (22.0%) died and 1562 (78.0%) survived; characteristics are described in **Suppl. Table 5**. Non-survivors had greater circulating levels of bio-ADM at baseline (105 pg/ml [62-219]) compared to survivors (56 pg/ml [30-114], p < 0.001). **Table 1** shows that baseline circulating bio-ADM was consistently higher in non-survivors versus survivors for each of the five main causes of ICU admission (p < 0.001 for all, except p = 0.002 in neurologic disorder). **Suppl. Figure 2** shows that the relationship between bio-ADM and mortality is non-linear and that the threshold at 70 pg/ml distinguishes between low and high risk of death; AUC is at 0.69 [0.66-0.72] and the threshold is at 75 pg/ml. Sensitivity analyses using a threshold at 75 pg/ml, or adjusting by SOFA or by SOFA and Charlson score showed similar associations between bio-ADM levels, organ dysfunction or death (**Suppl. Table 4**). **Suppl. Table 6** shows similar performance whether the threshold is at 70 or 75 pg/mL. **Figure 2B** further shows that baseline levels of bio-ADM were associated with an increased risk of 28-day mortality: unadjusted OR [95% CI]) at 3.03 [2.43-3.81] and adjusted OR for SAPS-II: 2.31 [1.82 – 2.92]) in our study cohort. **Figure 2B** further shows that high concentrations of bio-ADM were associated with an increased risk of 28-day mortality in all Charlson's score levels, as well as in septic shock, acute respiratory failure, out of hospital cardiac arrest and cardiogenic shock, but not with neurological disorders.

The Kaplan Meier curves of 28-day mortality further showed added prognostic value of the baseline bio-ADM on top of the SAPS-II score at admission. Indeed, low (< 49) SAPS-II score and low concentrations of baseline bio-ADM (< 70 pg/ml) showed low 28-day mortality (8.2%), whereas low SAPS-II and high bio-ADM were associated with a higher mortality (21.5%, **Figure 3**). High SAPS-II score and high bio-ADM were associated with the highest mortality (38.2%). OR of low SAPS-II and low bio-ADM versus low SAPS-II and high bio-ADM, high SAPS-II and low bio-ADM and high SAPS-II and high bio-ADM were (OR [95%]): 3.06 [2.11-4.45], 3.02 [2.09-4.37] and 6.91 [4.98-9.59] respectively (all p-values < 0.0001, **Figure 3**). Association between baseline bio-ADM and SAPS-II score is shown in **Suppl. Figure 3**.

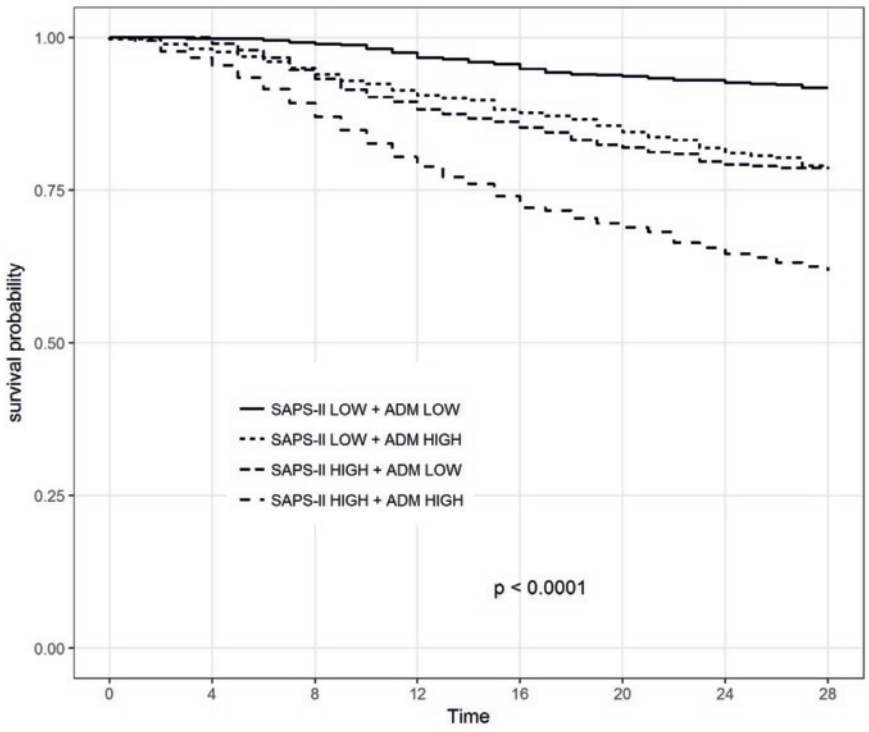

Figure 3. Kaplan-Meier survival curve according to SAPS-II and baseline bio-ADM. High level of ADM defined as ≥ 70 pg/mL, high levels of SAPS-II as ≥ 49. *Abbreviations: SAPS-II score, Simplified Acute Physiology Score II score.*

Discussion

In this analysis of the FROG-ICU study, a prospective multicenter study in the general ICU population, we show that elevated baseline levels of bio-ADM can be found in various critical illnesses associated with impaired vascular integrity or endothelial dysfunction. Our study further demonstrates that bio-ADM predicts the need for intensive therapies during ICU stay and had added prognostic value to SAPS-II to predict short-term outcome, in septic and in non-septic patients.

Our study shows that concentrations of bio-ADM at baseline were greatly increased in the general ICU population. Highest concentrations of bio-ADM were found in patients with septic and cardiogenic shock, which is in line with results from three previous studies[15,16,22]. In addition, our study shows elevated levels among cardiac arrest and acute respiratory failure patients but not in ICU patients admitted for neurological disorders. Overall, these levels of bio-ADM reflect the activation of systemic inflammatory pathways and the involvement of the endothelium, which has been shown to be a predominant source of ADM production previously[2,23].

Our study reveals a strong association with baseline bio-ADM concentrations and the need for inotrope(s) and/or vasopressor(s) treatment, renal replacement therapies and length of ICU stay in our general ICU population. Inclusion criteria included the presence of at least one dysfunctioning organ, which resulted in a homogeneous cohort of severely ill ICU patients. Thus, to predict a specific organ dysfunction, only patients without this specific organ dysfunction at admission were studied. Hence we confirmed associations between high bio-ADM level and organ dysfunction in septic patients, in line with previous findings[16] and more importantly we extend those associations to ICU patients admitted for non-septic causes. Effects of high levels of bio-ADM remain elusive. Some studies describe detrimental effects of high circulating bio-ADM that contribute to organ dysfunction including renal dysfunction, while other data suggest that high bio-ADM levels might protect the kidneys[17,22]. A trial was recently started to assess whether the modulation of the bio-ADM pathway by a specific non-neutralizing monoclonal anti-ADM antibody might improve organ support (AdrenOSS-2, NCT03085758). Of particular interest, our study also showed that baseline bio-ADM was able to predict the need for organ support in patients who did not need organ support at admission, but developed it later. Further studies are needed to elucidate the exact role of high bio-ADM as an early marker of organ dysfunction.

Our study also confirmed the association between circulating baseline bio-ADM and short-term outcome in sepsis and extended the knowledge in various non-septic causes of ICU admission. Our study further showed that the prognostic value of baseline bio-ADM exerts additive value to the SAPS-II severity score. Hence, our data suggest that vascular dysfunction may be very frequent in the general ICU population, even outside sepsis patients and could affect the outcome in a very large spectrum of critically ill patients.

Our study does have some limitations. First, only a single measurement of baseline bio-ADM was taken after inclusion, while previous studies have demonstrated added value of repeated bio-ADM measurements[15,16]. For future studies, an additional measurement a few days after admission might be of a great interest. Second, the association between bio-ADM level and outcome could be different according to the diagnosis on admission. This is why we repeated the analyses in the subgroups of sepsis patients compared to the non-septic ones. We did not further divide the non-septic patients into subgroups in order to avoid power issues. Also, note that we did not add cardiac biomarker (such as NT-proBNP and ST-2) to these data as they were described in FROG-ICU study[17] and were not the aim of this study. Furthermore, initiation of any organ support was determined by the treating physicians and not standardized among the 21 ICUs of FROG-ICU study. Another limitation was that doses of vasoactives drugs were not recorded. Finally, concerning the association between bio-ADM and organ function, only analyzed vascular and renal function were investigated in our study. Data on other organs (e.g. lungs, brain, etc.) were not assessed in the present study.

Conclusion

This analysis of the FROG-ICU study, a multicenter, prospective study in general ICU patients, showed that baseline levels of bio-ADM were increased in patients with sepsis, acute respiratory failure and out of hopsital cardiac arrest, but not with neurological disorders. Furthermore, high bio-ADM levels were strongly associated with the need for organ support, even in patients that did not have organ support at admission, and with 28-day mortality. Those data were included in the design of the ongoing biomarker-guided trial, AdrenOSS-2, that aims at improving/preventing organ function by modulation of the bio-ADM pathway in septic shock patients with high bio-ADM levels.

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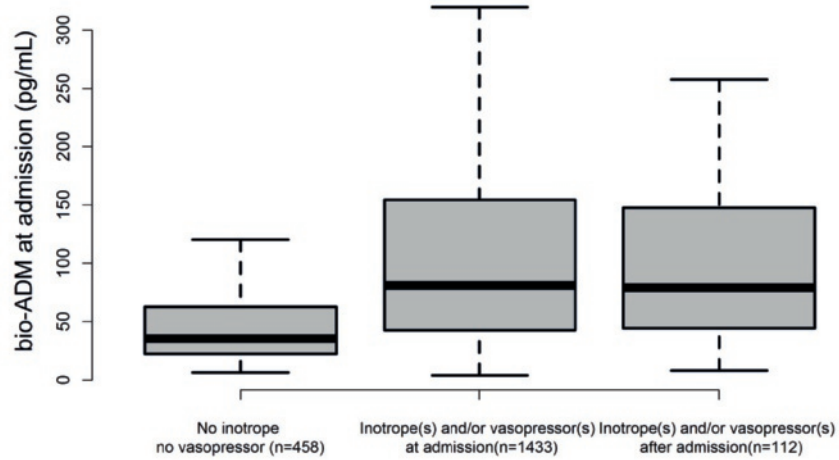
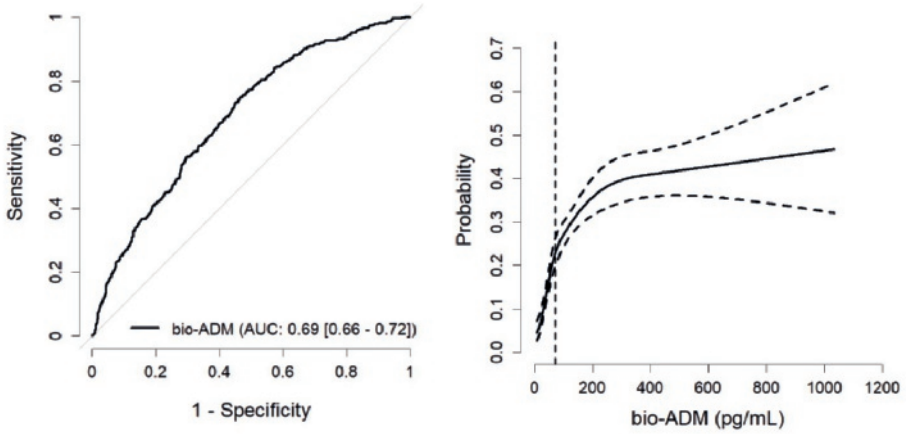
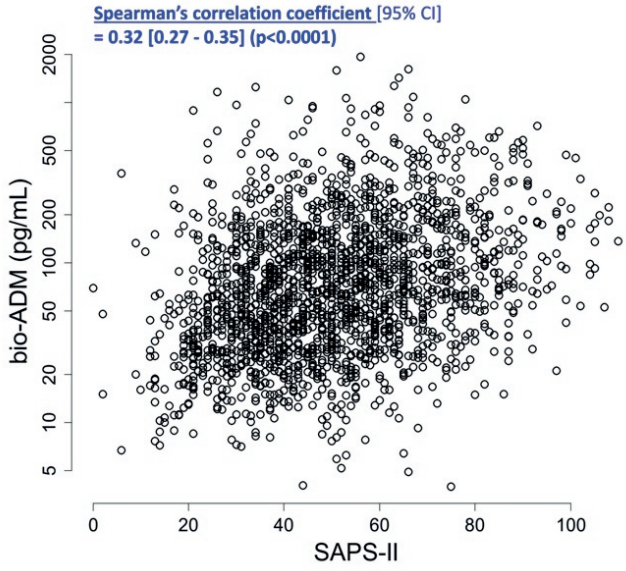


Suppl. Figure 1. Association between baseline bio-ADM and the need for inotrope(s) and/or vasopressor(s) during ICU stay.



Suppl. Figure 2. ROC curve of bio-ADM to predict 28-day mortality (left panel) and linearity of the association between bio-ADM level at admission and 28-day mortality (right panel). The vertical dotted line represents a bio-ADM level of 70 pg/mL.



Suppl. Figure 3. Correlation between baseline bio-ADM and SAPS-II score.

Suppl. Table 1. Baseline clinical characteristics according to low or high bio-ADM at ICU-admission.

	All patients (n=2003)	Bio-ADM < 70 pg/mL (n=1044)	Bio-ADM ≥ 70 pg/mL (n=959)	p-value
Age [year]	63 [51;74]	60 [47;71]	67 [56;77]	<0.0001
Male gender	1297 (65)	669 (64)	628 (66)	0.51
BMI [kg/m^2]	26 [23;31]	25 [22;29]	28 [25;32]	<0.0001
SAPS-II score	49 [36;63]	43 [32;57]	55 [41;68]	<0.0001
SOFA score	7 [4;10]	6 [3;8]	9 [6;11]	<0.0001
Charlson score	3 [1;5]	2 [1;4]	4 [2;5]	<0.0001
Main cardiovascular comorbidities				
Chronic heart failure	147 (7)	46 (4)	101 (11)	<0.0001
Hypertension	866 (43)	381 (37)	485 (51)	<0.0001
Diabetes mellitus	366 (18)	146 (14)	220 (23)	<0.0001
Dyslipidemia	393 (20)	183 (18)	210 (22)	0.013
Peripheral artery disease	198 (10)	77 (7)	121 (13)	<0.0001
Atrial fibrillation	215 (11)	81 (8)	134 (14)	<0.0001
Main non-cardiovascular comorbidities				
COPD	257 (13)	101 (10)	156 (16)	<0.0001
Chronic liver disease	156 (8)	59 (6)	97 (10)	0.00019
Active recent malignant tumors	274 (14)	120 (12)	154 (16)	0.0029
Depression	245 (12)	147 (14)	98 (10)	0.0086
Alcohol	346 (17)	180 (17)	166 (17)	0.96
Smoking	539 (27)	288 (28)	251 (26)	0.49
Main cause of admission				
Out of Hospital Cardiac Arrest	176 (9)	109 (10)	67 (7)	
Cardiogenic shock	137 (7)	64 (6)	73 (8)	
Septic shock	465 (23)	151 (15)	314 (33)	
Acute respiratory failure	381 (19)	236 (23)	145 (15)	
Acute neurological disorder	273 (14)	240 (23)	33 (3)	

Suppl. Table 1. continued.

	All patients (n=2003)	Bio-ADM < 70 pg/mL (n=1044)	Bio-ADM ≥ 70 pg/mL (n=959)	p-value
Status at admission				
Glasgow score	12 [3;15]	12 [3;15]	11 [3;15]	0.63
Hemoglobin [g/dL]	9.9 [8.9;11.4]	10.3 [9.2;11.8]	9.6 [8.7;10.8]	<0.0001
SBP [mmHg]	122 [108;139]	125 [110;143]	119 [106;135]	<0.0001
DBP [mmHg]	61 [53;70]	64 [55;74]	58 [51;67]	<0.0001
HR [bpm]	91 [78;106]	88 [75;104]	94 [81;109]	<0.0001
Temperature (°C)	37.2 (36.7 to 37.8)	37.3 (36.8 to 37.8)	37.2 (36.6 to 37.8)	<0.0001
Bicarbonates [mmol/L]	24 [21;26]	24 [22;27]	23 [20;26]	<0.0001
Lactate [mmol/L]	1.4 [1;1.9]	1.2 [0.9;1.6]	1.6 [1.1;2.4]	<0.0001
Platelets count [/ mm3]	164000 [99000;243500]	186000 [126000;263000]	131000 [79000;222000]	<0.0001
Prothrombin time [%]	69 [54;80]	74 [62;85]	63 [46;75]	<0.0001
White Blood Cells (/ mm3)	10900 [7500;16200]	10200 [7472.5;14900]	11770 [7800;17918]	<0.0001
CRP	124 [56;221]	101 [49;163]	164 [69;263]	<0.0001
Creatinine (µmol/L)	83 [59;150]	66 [52;87]	134 [83;212]	<0.0001
Urea (mmol/L)	8.5 [5.3;14.1]	6.6 [4.4;10]	11.7 [7.5;17.9]	<0.0001
NGAL	211 [97;509]	112.5 [68;188]	483 [253;873]	<0.0001
PCT	1.1 [0.3;5.6]	0.5 [0.2;1.6]	3.5 [0.9;13.1]	<0.0001
AST	53 [31;119]	45 [28;89]	64 [37;152]	<0.0001
ALT	40 [22;92]	38 [21;79]	42 [22;109]	0.0082
Bilirubine (mmol/L)	13 [8;28]	11 [7;19]	17 [9;38]	<0.0001
Bio-ADM	66.6 [34.6;136.3]	35.5 [23.9;50]	141.6 [96.5;223.9]	<0.0001
In-ICU management				
In-ICU LOS (days)	13 [7;21]	11 [7;19]	14 [8;24]	<0.0001
RRT	467 (23)	103 (10)	364 (38)	<0.0001
Inotrope(s) and/or vasopressor(s)	1545 (77)	686 (66)	859 (90)	<0.0001
28-day mortality	441 (22)	138 (13)	303 (32)	<0.0001

Data are presented as median [IQR] or mean (SD). High level of ADM is defined as ≥ 70 pg/mL. *Abbreviations: BMI: Body Mass Index, SAPS-II score: Simplified Acute Physiology Score II score, SOFA score: Sequential Organ Failure Assessment score, COPD: Chronic Obstruction Pulmonary Disease, ACR: Arrest Cardiac Respiratory, GCS: Glasgow Coma Scale, Hb: Hemoglobin, SBP: Systolic Blood Pressure, DBP: Diastolic Blood Pressure, HR: Heart rate, ICU LOS: Intensive Care Units Length of Stay, RRT: Renal Replacement Therapy, RBC: Red Blood Cell, FFP: Frozen Plasma.*

Suppl. Table 2. Bio-ADM value with regard to renal function at admission.

	Bio-ADM at admission	p-value
Chronic kidney disease		<0.0001
No	60.9 (31.8 to 121)	
Yes	130 (78.1 to 225.1)	
eGFR at admission		<0.0001
<60	125.6 (75.1 to 222.7)	
60-90	57.9 (36.7 to 105.8)	
>90	36.1 (22.3 to 63.9)	

Abbreviations: eGFR, estimated glomerular filtration rate.

Suppl. Table 3. Primary and secondary endpoints in septic shock and non-septic shock subgroups

	All included patients	Septic shock patients	Non-septic shock patients
	OR of high ADM [95% CI]	OR of high ADM [95% CI]	OR of high ADM [95% CI]
Need for RRT			
All patients	4.89 [3.82 - 6.26]	2.83 [1.77 - 4.53]	5.45 [4.06 - 7.31]
eGFR at adm > 60 mL/min	3.14 [2.10 - 4.71]	1.93 [0.93 - 3.98]	3.2 [1.95 - 5.25]
eGFR at adm > 90 mL/min	3.98 [2.21 - 7.18]	8.46 [1.78 - 40.34]	3.06 [1.52 - 6.14]
Need for vasopressor			
All patients	3.64 [2.83 - 4.67]	2.75 [1.12 - 6.72]	3.25 [2.49 - 4.24]
No vasopressor at admission	3.70 [2.36 - 5.81]	4.96 [0.96 - 25.74]	3.21 [1.98 - 5.22]
Prolonged ICU LOS			
All patients	1.85 [1.49 - 2.29]	1.58 [0.99 - 2.5]	2.00 [1.56 - 2.56]
28-day mortality	2.31 [1.82 - 2.92]	3.19 [1.81 - 5.61]	2.16 [1.65 - 2.82]

High level of ADM is defined as ≥ 70 pg/mL. *Abbreviations: eGFR, estimated glomerular filtration rate; ICU LOS, intensive care unit length of stay; RRT, renal replacement therapy.*

Suppl. Table 4. Sensitivity analyses of the association between bio-ADM at ICU admission and outcome.

	Using a cut-off value of 75 pg/mL bio-ADM	With adjustment on SOFA score at admission	With adjustment on SOFA at admission and Charlson's score
28-day mortality	2.27 [1.8 - 2.87]	2.52 [1.93 - 3.29]	2.03 [1.54 - 2.68]
RRT			
All patients	4.96 [3.89 - 6.32]	4.92 [3.67 - 6.59]	4.72 [3.5 - 6.36]
eGFR at adm > 90 mL/min	3.39 [2.26 - 5.08]	3.14 [1.9 - 5.18]	2.95 [1.77 - 4.91]
eGFR at adm > 60 mL/min	4.14 [2.29 - 7.48]	3.92 [1.88 - 8.16]	3.62 [1.73 - 7.58]
Vasopressor and/or inotropes			
All patients	3.53 [2.74 - 4.56]	4.38 [3.17 - 6.06]	3.9 [2.8 - 5.44]
No vaso at admission	3.72 [2.36 - 5.87]	4.28 [2.43 - 7.54]	3.97 [2.21 - 7.12]

Abbreviations: adm, admission; eGFR, estimated glomerular filtration rate; RRT, renal replacement therapy; vaso, vasopressor(s) and/or inotrope(s).

Suppl. Table 5. Baseline clinical characteristics according to 28-day ICU mortality.

	All patients (n = 2003)	Alive patients (n = 1562)	Dead patients (n = 841)	p-value
Age [year]	63 [51;74]	61 [49;72]	71 [61;79]	<0.0001
Male gender	1297 (65)	993 (64)	304 (69)	0.037
BMI [kg/m2]	26 [23;31]	26 [23;31]	27 [24;31]	0.24
SAPS-II score	49 [36;63]	46 [34;59]	60 [46;74]	<0.0001
SOFA score	7 [4;10]	6 [4;9]	9 [7;12]	<0.0001
Charlson score	3 [1;5]	3 [1;4]	4 [3;6]	<0.0001
Main cardiovascular comorbidities				
Chronic heart failure	147 (7)	99 (6)	48 (11)	0.0012
Hypertension	866 (43)	623 (40)	243 (55)	<0.0001
Diabetes mellitus	366 (18)	266 (17)	100 (23)	0.0065
Dyslipidemia	393 (20)	305 (20)	88 (20)	0.83
Peripheral artery disease	198 (10)	143 (9)	55 (13)	0.039
Atrial fibrillation	215 (11)	152 (10)	63 (14)	0.0062
Main non-cardiovascular comorbidities				
COPD	257 (13)	186 (12)	71 (16)	0.02
Chronic liver disease	156 (8)	97 (6)	59 (13)	<0.0001
Active recent malignant tumors	274 (14)	190 (12)	84 (19)	0.0002
Depression	245 (12)	201 (13)	44 (10)	0.1
Alcohol	346 (17)	265 (17)	81 (18)	0.49
Smoking	539 (27)	445 (29)	94 (21)	0.0028
Main cause of admission				
Out of Hospital Cardiac Arrest	176 (9)	116 (7)	60 (14)	
Cardiogenic shock	137 (7)	103 (7)	34 (8)	
Septic shock	465 (23)	337 (22)	128 (29)	
Acute respiratory failure	381 (19)	298 (19)	83 (19)	
Acute neurological disorder	273 (14)	242 (16)	31 (7)	
Status at admission				
GCS	11.5 [3;15]	13 [3;15]	6 [3;14]	<0.0001
Hemoglobin [g/dL]	9.9 [8.9;11.4]	10 [9;11.4]	9.8 [8.8;11.2]	0.018
SBP [mmHg]	122 [108;139]	123 [110;140]	117 [104;133]	<0.0001
DBP [mmHg]	61 [53;70]	62 [55;71]	56 [50;65]	<0.0001
HR [bpm]	91 [78;106]	90 [77;105]	95 [82;111]	<0.0001
Temperature (°C)	37.2 (36.7 to 37.8)	37.3 (36.8 to 37.8)	37 (36.4 to 37.7)	<0.0001
Bicarbonates [mmol/L]	24 [21;26]	24 [21;27]	23 [20;25]	<0.0001
Lactate [mmol/L]	1.4 [1;1.9]	1.3 [0.9;1.8]	1.7 [1.1;2.6]	<0.0001

Suppl. Table 5. continued.

	All patients (n = 2003)	Alive patients (n = 1562)	Dead patients (n = 841)	p-value
Platelets count [/mm3]	164000 [99000;243500]	169000 [106000;252000]	136000 [80000;218000]	<0.0001
Prothrombin time [%]	69 [54;80]	71 [57;81]	59 [44;74]	<0.0001
White Blood Cells (/mm3)	10900 [7500;16200]	10500 [7400;15498]	12560 [8100;18543]	<0.0001
CRP	124 [56;221]	124 [58;217]	121 [52;232]	0.91
Creatinine (µmol/L)	83 [59;150]	78 [57;134]	120 [75;193]	<0.0001
Urea (mmol/L)	8.5 [5.3;14.1]	8 [5.1;13.1]	11.1 [7.1;17.4]	<0.0001
NGAL	211 [97;509]	175 [86;418]	415 [182;877]	<0.0001
PCT	1.1 [0.3;5.6]	0.9 [0.3;4.3]	2.7 [0.7;10.2]	<0.0001
AST	53 [31;119]	49 [29;103]	76 [40;210]	<0.0001
ALT	40 [22;92]	39 [21;86]	48.5 [26;148]	<0.0001
Bilirubine (mmol/L)	13 [8;28]	12 [8;24]	16 [10;42]	<0.0001
Bio-ADM	66.6 [34.6;136.3]	56.4 [30;114.7]	105.3 [61.9;218.9]	<0.0001
In-ICU management				
In-ICU LOS (days)	13 (7;21)	13 (7;24)	11 (7;16)	<0.0001
RRT	467 (23)	311 (20)	156 (35)	<0.0001
Inotrope / vasopressor	1545 (77)	1154 (74)	391 (89)	<0.0001
28-day mortality	441 (22)	0 (0)	441 (100)	0

Abbreviations: adm, admission; BMI, Body Mass Index; COPD, Chronic Obstructive Pulmonary Disease; DBP, Diastolic Blood Pressure; GCS, Glasgow Coma Scale; HR, Heart Rate; eGFR, estimated Glomerular Filtration Rate; RRT, Renal Replacement Therapy; SBP, Systolic Blood Pressure; SOFA, Sequential Organ Failure Assessment.

Suppl. Table 6. Performance of the cut-off values considered in the study

	Value of the cut-off	Sensitivity	Specificity	NPV	PPV
Predetermined cut-off	70 pg/mL	69%	68%	87%	32%
ROC curve derived cut-off (closest top-left approach)	75 pg/mL	67%	60%	86%	32%

Abbreviations: NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating characteristic.

Chapter 8

Circulating adrenomedullin estimates survival and reversibility of organ failure in sepsis: the prospective observational multinational Adrenomedullin and Outcome in Sepsis and Septic Shock 1 (AdrenOSS-1) study

Alexandre Mebazaa, Christopher Geven, Alexa Hollinger, Xavier Wittebolle, Benjamin Glen Chousterman, Alice Blet, Etienne Gayat, Oliver Hartmann, Paul Scigalla, Joachim Struck, Andreas Bergmann, Massimo Antonelli, Albertus Beishuizen, Jean-Michel Constantin, Charles Damoisel, Nicolas Deye, Salvatore Di Somma, Thierry Dugernier, Bruno François, Stephane Gaudry Vincent Huberlant, Jean-Baptiste Lascarrou, Gernot Marx, Emmanuelle Mercier, Haikel Oueslati, Peter Pickkers, Romain Sonneville, Matthieu Legrand, Pierre-François Latterre and AdrenOSS-1 study investigators

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Abstract

Background

Adrenomedullin (ADM) regulates vascular tone and endothelial permeability during sepsis. Levels of circulating biologically active ADM (bio-ADM) show an inverse relationship with blood pressure and a direct relationship with vasopressor requirement. In the present prospective observational multinational Adrenomedullin and Outcome in Sepsis and Septic Shock 1 (AdrenOSS-1) study, we assessed relationships between circulating bio-ADM during the initial intensive care unit (ICU) stay and short-term outcome in order to eventually design a biomarker-guided randomized controlled trial.

Methods

AdrenOSS-1 was a prospective observational multinational study. The primary outcome was 28-day mortality. Secondary outcomes included organ failure as defined by Sequential Organ Failure Assessment (SOFA) score, organ support with focus on vasopressor/inotropic use, and need for renal replacement therapy. AdrenOSS-1 included 583 patients admitted to the ICU with sepsis or septic shock.

Results

Circulating bio-ADM levels were measured upon admission and at day 2. Median bio-ADM concentration upon admission was 80.5 pg/ml [IQR 41.5–148.1 pg/ml]. Initial SOFA score was 7 [IQR 5–10], and 28-day mortality was 22%. We found marked associations between bio-ADM upon admission and 28-day mortality (unadjusted standardized HR 2.3 [CI 1.9–2.9]; adjusted HR 1.6 [CI 1.1–2.5]) and between bio-ADM levels and SOFA score ($p<0.0001$). Need of vasopressor/inotrope, renal replacement therapy, and positive fluid balance were more prevalent in patients with a bio-ADM>70 pg/ml upon admission than in those with bio-ADM≤70 pg/ml. In patients with bio-ADM>0 pg/ml upon admission, decrease in bio-ADM below 70 pg/ml at day 2 was associated with recovery of organ function at day 7 and better 28-day outcome (9.5% mortality). By contrast, persistently elevated bio-ADM at day 2 was associated with prolonged organ dysfunction and high 28-day mortality (38.1% mortality, HR 4.9, 95% CI 2.5–9.8).

Conclusions

AdrenOSS-1 shows that early levels and rapid changes in bio-ADM estimate short-term outcome in sepsis and septic shock. These data are the backbone of the design of the biomarker-guided AdrenOSS-2 trial.

Introduction

Adrenomedullin (ADM) is a free circulating peptide with potent vascular properties, including beneficial effects on endothelial barriers at physiological levels. ADM has previously been described as a "double-edged sword" in sepsis[1] because high levels of ADM induce vasodilation and hypotension[2-4] on one hand while reinforcing the endothelial barrier and improving outcome on the other[5-10]. The potential of ADM as a prognostic biomarker has previously been studied in critically ill patients, often by measuring the inactive midregional pro-ADM[11,12], or recently by direct measurement of the bioactive form of ADM (bio-ADM)[13,14]. It has been shown repeatedly that bio-ADM greater than 70 pg/ml is associated with worse outcome[13,14].

On the basis of previous results, we tested the hypothesis that modulating the ADM pathway in patients with high levels of circulating bio-ADM may improve short-term outcome in sepsis. Adrecizumab, a monoclonal anti-ADM antibody, has been shown to improve organ function in preclinical settings[15]. In order to design a human trial in whivach we would administer adrecizumab based on levels of bio-ADM, we needed to assess the relationship between initial levels of bio-ADM and short-term outcome in sepsis and in septic shock patients.

In the Adrenomedullin and Outcome in Sepsis and Septic Shock 1 (AdrenOSS-1) study, we investigated whether the initial plasma concentration of bio-ADM (on intensive care unit [ICU] admission and after 48 h) may provide insight into 28-day survival and the recovery of organ function.

Methods

Study design

AdrenOSS-1 was a European prospective observational study. Twenty-four centers in five countries (France, Belgium, The Netherlands, Italy, and Germany) contributed to the trial achievement of 583 enrolled patients. Patients were recruited from June 2015 to May 2016. The study protocol was approved by the local ethics committees and was conducted in accordance with Directive 2001/20/EC, as well as good clinical practice (International Conference on Harmonization Harmonized Tripartite Guideline version 4 of May 1, 1996, and decision of November 24, 2006) and the Declaration of Helsinki.

The study enrolled patients aged 18 years and older who were (1) admitted to the ICU for sepsis or septic shock or (2) transferred from another ICU in the state of sepsis and septic shock within less than 24 h after admission. Included patients were stratified by severe sepsis and septic shock based on definitions for sepsis and organ failure from 2001[16]. In the present article, the term "sepsis" refers to the updated definition of Sepsis-3[17]. Concerning septic shock, most data presented in this article are based on the former definition[16], except for the confirmatory analyses presented in the last paragraph of the "Results" section, for which the new Sepsis-3 definition of septic shock was used[17].

Exclusion criteria were pregnancy, vegetative coma, and participation in an interventional trial in the preceding month. Informed consent was obtained from all patients or their lawful representatives prior to enrollment in the study. Patients were treated according to local practice, and treatments as well as procedures were registered.

The primary endpoint was 28-day mortality. Secondary endpoints concerned organ failure (as defined by the Sequential Organ Failure Assessment [SOFA] score) and organ support, vasopressor/inotrope use, fluid balance, and use of renal replacement therapy (RRT), as well as validation of the previously identified cutoff value of 70 pg/ml[14]. The latter was identified as the optimal screening cutoff for AdrenOSS-2, an ongoing proof-of-concept and dose-finding phase II trial assessing adrecizumab (an antibody modulating circulating bio-ADM) in patients with early septic shock (NCT03085758). The relationship between cardiovascular SOFA subscore and bio-ADM, being a biomarker of vascular dysfunction, was evaluated.

Collection of patient data
Upon admission, demographics (age, sex), body mass index, presence of septic shock, type of ICU admission, organ dysfunction scores (SOFA, Acute Physiologic Assessment and Chronic Health Evaluation II [APACHE II]), origin of sepsis, preexisting comorbidities (i.e., treated within the last year), past medical history, laboratory values, and organ support were recorded, and blood was drawn for measurement of bio-ADM and other markers.

After patient enrollment, the following data were collected daily during the first week: SOFA score, antimicrobial therapies, fluid balance, ventilation status, Glasgow Coma Scale score, central venous pressure, need for RRT, invasive procedures for sepsis control, and vasopressor/inotrope treatment. Moreover, discharge status and mortality were recorded on day 28 after ICU admission.

Sample collection
Blood for the central laboratory was sampled within 24 h after ICU admission and on day 2 (mean 47 h, SD 9 h) after the first sample. Samples were subsequently processed and stored at – 80 °C before transfer to the central laboratory for blinded bio-ADM analysis organized by the study sponsor (sphingotec GmbH, Hennigsdorf, Germany). Routine analyses (e.g., partial pressure of arterial oxygen, lactate) were performed by the local laboratories.

Bio-ADM measurement
Bio-ADM was measured using a recently developed immunoassay provided by sphingotec GmbH. For details and design principles on the assay, see publications by Marino et al.[14] and Weber et al.[18]. The analytical assay sensitivity was 2 pg/ml.

Statistical analyses
Results are presented as number and percentage, mean and SD, or median and IQR, depending on their distribution. Group comparisons for continuous variables were performed using the Kruskal-Wallis test, and appropriate post hoc tests were applied if necessary. Categorical data were compared using the chi-square test with simulated p-values using 2000 replicates. Biomarker data were log-transformed if necessary. Cox proportional hazards regression was used to analyze the effect of risk factors on survival in uni- and multivariable analyses. The assumptions of proportional hazards were tested for all

variables. For continuous variables, HRs were standardized to describe the HR for a biomarker change of one IQR. CIs (95% CI) for risk factors and significance levels for chi-square (Wald) test are given. The predictive value of each model was assessed by the model likelihood ratio chi-square statistic. The concordance index (C index) is given as an effect measure. It is equivalent to the concept of AUC adopted for binary outcome. For multivariable models, a bootstrap-corrected version of the C index is given. To test for added predictive value, we used the likelihood ratio chi-square test for nested models to assess whether bio-ADM adds predictive value to a clinical model or a risk score. Survival curves plotted by the Kaplan-Meier method using quartiles or predefined cut points (70 pg/ml) of bio-ADM were used for illustrative purposes. ROC curve analysis was applied for 28-day mortality to determine the optimal Youden cutoff in this cohort.

A two-sided p-value of 0.05 was considered statistically significant. All analyses were performed using R version 2.5.1 (http://www.r-project.org, library Design, Hmisc, ROCR) and IBM SPSS Statistics version 22.0 software (IBM, Armonk, NY, USA).

Results

A total of 583 patients were included in the AdrenOSS-1 study. Patient characteristics, organ dysfunction scores, physiological and laboratory values, organ support upon admission, and outcome parameters are presented in **Table 1**. The median bio-ADM level at admission was 80.5 pg/ml [IQR 41.6–148.1] in our studied patients; 55.9% had bio-ADM level greater than 70 pg/ml at admission, and 44.1% had a bio-ADM less than 70 pg/ml. Of note, patients with septic shock had a significantly higher bio-ADM concentration at admission than patients with sepsis (114.4 [62.6–214.5] versus 57.5 pg/ml [31.2–101.5], $p < 0.0001$).

Bio-ADM levels and mortality

Over the 28-day follow-up period, 127 patients (22%) died: 33 with sepsis and 94 with septic shock.

In a Cox proportional hazards model adjusted for age, gender, comorbidities (cardiac and noncardiac), lactate, and diagnosis (sepsis, septic shock), bio-ADM concentration at admission was independently associated with 28-day mortality in the studied population (added chi-square 12.2, $p = 0.0005$; adjusted standardized HR 1.6 [95% CI 1.1–2.5], $p = 0.0004$) (**Table 2**).

Noticeably, the C index for prediction of 28-day mortality for bio-ADM at admission was 0.688 (95% CI 0.642–0.733, chi-square 54.8, $p < 0.0001$) in the univariate Cox regression. C indexes for lactate, SOFA, and APACHE II were 0.720 (95% CI 0.672–0.768), 0.728 (95% CI 0.680–0.777), and 0.701 (95% CI 0.657–0.746), respectively (all $p < 0.0001$). A multivariate model further demonstrated that bio-ADM had added value on top of APACHE II or SOFA score (added chi-square 24.4 [$p < 0.0001$] and 10.2 [$p = 0.0014$], respectively) (**Table 2**) when used as a continuous variable.

With the predefined cutoff value of 70 pg/ml, Kaplan-Meier analysis confirmed predictive value of bio-ADM for 28-day mortality in all studied patients (**Figure S1**) and in subgroups of sepsis and septic shock (**Figure 1a and b**). Patient characteristics for high and low bio-ADM levels are illustrated in **Table 1**, and characteristics for survivors versus nonsurvivors are provided in **Table S1**. The optimal Youden cutoff in all patients was 101.9 pg/ml (sensitivity 67.7%, specificity 67.3%). In septic shock, the optimal Youden cutoff was 99.1 pg/ml (sensitivity 71.3%, specificity 52.3%), and in severe sepsis it was 101.9 pg/ml (sensitivity 57.6%, specificity 78.6%). This compares with a sensitivity of 77.2% and specificity of 48.9% in all patients for the predefined bio-ADM cutoff of 70 pg/ml.

Additionally, we assessed outcome in relation to bio-ADM changes in the initial 48 h using time-dependent Cox regression. Bio-ADM trajectory over the initial 48 h after study inclusion improved prediction of 28-day survival in the overall population (added chi-square 25.8, $p < 0.0001$) (**Table 2**; **Fig. 2**, **Figure S2**) and was independent of time-dependent lactate or SOFA score evaluation (**Table 2**). Patients were divided into four groups based on baseline and day 2 bio-ADM concentrations and under implementation of the cutoff value of 70 pg/ml: remaining low (low-low, LL), high-to-low (HL), low-to-high (LH), and remaining high (high-high, HH). Patient characteristics of these subgroups are presented in **Table S2**.

In patients admitted with high bio-ADM upon admission, those with decreased bio-ADM towards normal values within the first 48 h (HL group) had a similar 28-day mortality to the LL group (HL 9.5%, LL 10.5%) and a more favorable outcome than patients whose bio-ADM remained high (HH group) or became high (LH group) (28-day mortality of 38.1% and 38.2%) (**Table S2**).

Table 1. Patient characteristics.

Patient characteristics	All	Bio-ADM < 70 pg/ml at admission	Bio-ADM > 70 pg/ml at admission	p-value*	n
Epidemiological data	n=538	n=257	n=326		
Bio-ADM at admission (pg/ml)	80.5 [41.5-148.0]	36.9 [27.1-51.0]	136.7 [97.6-241.0]	<0.0001	
Age (year)	66 [55-76]	64 [53-75]	67 [58-76]	0.0052	
Males (No. %)	364 (62.4)	171 (66.5)	193 (59.2)	0.0837	
Body Mass Index (kg/m2)	25.7 [22.9-30.1]	25.0 [22.3-28.4]	26.7 [23.2-31.6]	0.0013	
Septic shock at admission (yes)	293 (50.3)	84 (32.7)	209 (64.1)	<0.0001	
Type of ICU admission				<0.0001	
Medical	473 (81.1)	230 (89.5)	243 (74.5)		
Surgical – emergency procedure	93 (16)	21 (8.2)	72 (22.1)		
Surgical – elective procedure	17 (2.9)	6 (2.3)	11 (3.4)		
Origin of sepsis				<0.0001	
Lung	218 (37.4)	129 (50.2)	89 (27.3)		
Blood stream	90 (15.4)	31 (12.1)	59 (18.1)		
Urinary tract	62 (10.6)	10 (3.9)	52 (16)		
Catheter	29 (5)	9 (3.5)	20 (6.1)		
Peritonitis	31 (5.3)	12 (4.7)	19 (5.8)		
Endocarditis	31 (5.3)	12 (4.7)	19 (5.8)		
Bile duct infection	8 (1.4)	2 (0.8)	6 (1.8)		
CNS	4 (0.7)	4 (1.6)	0 (0)		
Skin and soft tissue	10 (1.7)	9 (3.5)	1 (0.3)		
Gynaecologic	2 (0.3)	1 (0.4)	1 (0.3)		
Other	98 (16.8)	38 (14.8)	60 (18.4)		
Medical history**					
Any cardiac comorbidity (yes)	400 (68.6)	147 (57.2)	253 (77.6)	<0.0001	
Chronic heart failure (yes)	60 (10.3)	19 (7.4)	41 (12.6)	0.0544	
Hypertension (yes)	293 (50.3)	105 (40.9)	188 (57.7)	<0.0001	
Diabetes Mellitus (yes)	160 (27.4)	57 (22.2)	103 (31.6)	0.0150	
Any non-cardiac comorbidity (yes)	414 (71)	167 (65)	247 (75.8)	0.0058	
Chronic renal disease (yes)	76 (13.0)	19 (7.4)	57 (17.5)	0.0004	
Active/recent malignant tumors (yes)	124 (21.3)	34 (13.2)	90 (27.6)	<0.0001	
Smoking (active, yes)	117 (20.1)	63 (24.5)	54 (16.6)	0.0302	
COPD (yes)	89 (15.3)	37 (14.4)	52 (16.0)	0.6421	
Any chronic medications (yes)	371 (63.6)	138 (53.7)	233 (71.5)	<0.0001	
Immunosuppressive therapy (yes)	46 (7.9)	11 (4.3)	35 (10.7)	0.0066	
Physiological values at admission					
Temperature (°C)	37.2 [36.4-38.2]	37.4 [36.6-38.2]	37.1 [36.2-38.1]	0.0034	
Mean blood pressure (mmHg)	75 [64-90]	81 [69-95]	72 [60-85]	<0.0001	
Heart rate (bpm)	104 [90-119]	100 [86-116]	105 [94-121]	0.0013	
Central venous pressure (mmHg)	8 [5-13]	8 [5-13]	10 [6-14]	0.2419	

Table 1. continued.

Patient characteristics	All	Bio-ADM < 70 pg/ml at admission	Bio-ADM > 70 pg/ml at admission	p-value*	n
Glasgow score	15 [14-15]	15 [14-15]	15 [14-15]	0.8161	
Fluid balance (ml)	1928 [592-3552]	1425 [500-2699]	2311 [764-4202]	<0.0001	
Urine output for 24 hours (ml)	1000 [450-1900]	1276 [650-2050]	800 [300-1650]	<0.0001	
PaO2/FiO2	228 [137-340]	233.5 [140-360]	223 [137-337]	0.4995	
Laboratory values at admission					
Lactate (mmol/l)	1.4 [1.0-2.2]	1.1 [0.8-1.6]	1.8 [1.2-2.7]	<0.0001	n=562
Arterial pH	7.38 [7.3-7.44]	7.42 [7.36-7.46]	7.36 [7.27-7.42]	<0.0001	
Bilirubin (umol/l)	11 [6-19]	10 [6.5-17]	12 [6-21]	0.1360	
Platelets (10^9/l)	190 [121-275]	196 [136-279]	181 [104-271]	0.0583	
Creatinine (mg/dl)	1.4 [0.9-2.2]	1 [0.7-1.4]	1.8 [1.2-2.9]	<0.0001	
BUN or Urea (mg/dl)	61 [37-107]	44 [28-69]	80 [50-127]	<0.0001	
Hematocrit (%)	34 [29-38]	35 [30-38]	34 [29-38]	0.1010	
White blood count (per mm^3)	12525 [7200-18585]	13000 [8475-18075]	12025 [5942-19025]	0.0547	
Troponin T, maximum on day 1	42 [18-158]	29 [14-124]	55 [25-176]	0.0230	n=153
Troponin I, maximum on day 1	69 [20-246]	40 [11-228]	99 [40-289]	0.0049	n=186
PCT, maximum on day 1 (ng/ml)	11.4 [1.9-49.8]	3.9 [0.9-19.5]	24 [6-84]	<0.0001	n=330
PCT, central lab (ng/ml)	10.2 [2.3-34.3]	3.7 [0.8-13.0]	18.2 [6.0-52.7]	<0.0001	n=583
BNP, maximum on day 1	257 [102-723]	187 [61-388]	473 [147-1154]	0.0004	n=131
NT-proBNP, maximum on day	4382 [1525-11565]	2170 [497-6633]	6116 [2816-15431]	0.0001	n=117
Organ support at admission					
Mechanical ventilation				0.0739	
Invasive	219 (37.6)	85 (33.1)	134 (41.1)		
Non-invasive	131 (22.5)	67 (26.1)	64 (19.6)		
None	233 (40.0)	105 (40.9)	128 (39.3)		
Renal replacement therapy (yes)	49 (8.4)	8 (3.1)	41 (12.6)	0.0001	
Vasopressors/inotropes at admission (yes)	349 (59.9)	109 (42.4)	240 (73.6)	<0.0001	
Organ dysfunction scores					
SOFA (points)	7 [5-10]	5 [3-8]	8 [6-11]	<0.0001	n=509
APACHE 2 (points)	15 [11-20]	14 [9-17]	18 [13-22]	<0.0001	
Length of stay (days)					
ICU	5 [2-10]	4 [2-8]	5 [2-10]	0.0554	
Mortality (%)					
28-day, deaths	127 (21.8)	30 (11.7)	97 (29.8)	<0.0001	
90-day, deaths	166 (28.5)	41 (16)	125 (38.3)	<0.0001	

* p-value from non-parametric Kruskal-Wallis or Chi2 test.
** most common comorbidities reported individually.
Abbreviations: APACHE, acute physiology and chronic health evaluation; Bio-ADM, bioactive adrenomedullin; BNP, brain-derived natriuretic peptide; BUN, blood urea nitrogen; CNS, central nervous system; COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; PCT, procalcitonin; SOFA, sequential organ failure assessment; NT-proBNP, N-terminal brain natriuretic peptide.

Table 2. Association between bio-ADM and 28-day mortality.

Variables	Chi²	Added chi²	P-value (added value)	Std. HR bio-ADM	p-value
Bio-ADM (univariate)	54.8			2.3 [1.9-2.9]	<0.0001
... adj. for SOFA at admission	85.1	10.2	0.0014	1.6 [1.2-2.1]	0.0014
... adj. for APACHE 2 at admission	88.9	24.4	<0.0001	1.9 [1.5-2.4]	<0.0001
... adj. for covariates	132.1	12.2	0.0005	1.6 [1.1-2.5]	0.0004
Bio-ADM (time-dependent Cox)	80.6	25.8	<0.0001	2.5 [2.1-3.1]	<0.0001
... adj. for SOFA at admission	89.3	11.5	0.0007	1.8 [1.4-2.2]	<0.0001
... adj. for APACHE 2 at admission	108.4	19.5	<0.0001	2.1 [1.7-2.6]	<0.0001
... adj. for SOFA (t-d*)	101.0	7.9	0.0049	1.5 [1.1-2.0]	0.0048
... adj. for lactate (t-d*)	138.0	35.7	<0.0001	1.9 [1.5-2.3]	<0.0001

Results are from uni- (chi-square), multi- (added chi-square), and *time-dependent Cox regression analysis. *Time-dependent analysis includes measurements observed at baseline and day 2. n = 562 for covariates (i.e., age, gender, comorbidities [cardiac and noncardiac], diagnosis [sepsis, septic shock], lactate) model due to missing data for time-dependent lactate, and n = 509 for models including *time-dependent SOFA score. *Abbreviations: APACHE, Acute Physiology and Chronic Health Evaluation II; bio-ADM, Bioactive adrenomedullin; SOFA, Sequential Organ Failure Assessment.*

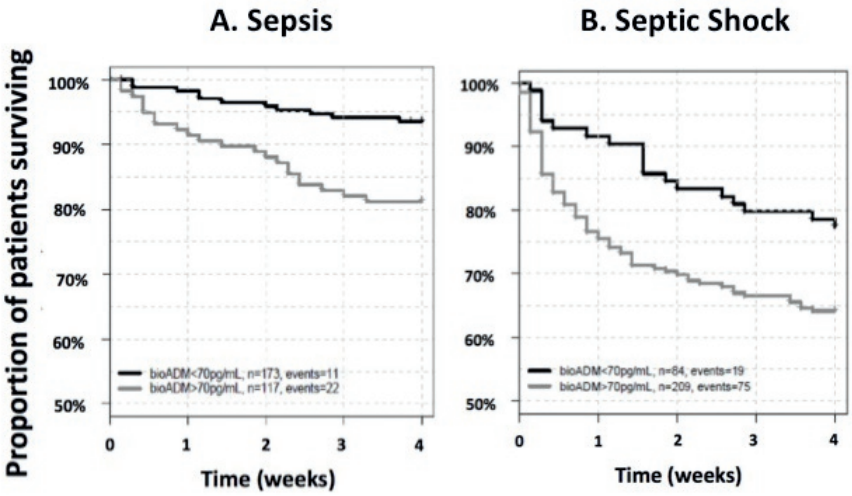

Figure 1. Twenty-eight-day Kaplan-Meier survival curves of low versus high biologically active adrenomedullin at admission, based on a cutoff value of 70 pg/ml, in (**A**) sepsis, and (**B**) septic shock patients.

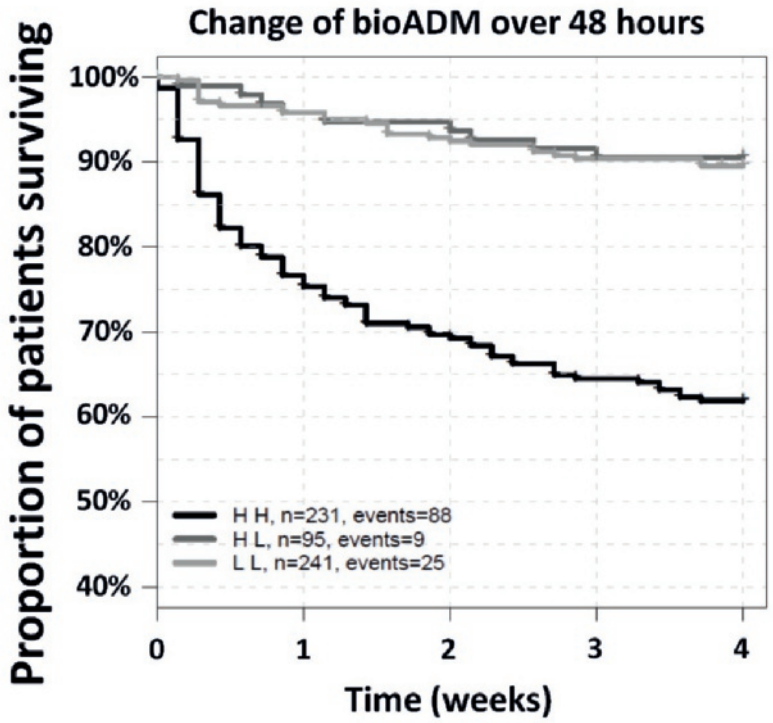

Figure 2. Association between the changes of biologically active adrenomedullin (bio-ADM) levels over 48 h and mortality. HR between high-high (HH) (levels of bio-ADM remained high) and high-low (HL) (levels of bio-ADM declining over 48 h) 4.9 (95% CI 2.5–9.8; HR of LL 1.1 [0.52–2.4]). Only a small number (n = 16, 2.7%; 28-day survival rate 68.8%) of patients who presented with a low bio-ADM concentration upon admission had higher bio-ADM level on day 2 (low-high (LH) group), which is why this group is not represented in the figure.

Bio-ADM levels and organ dysfunction
Bio-ADM levels upon admission correlated with the initial SOFA score in all studied patients ($n = 509$, $r = 0.49$, $p < 0.0001$) (**Figure S3**). SOFA score was higher in patients in septic shock than in those in sepsis, and for each group in patients with high initial bio-ADM (**Figure S4**). **Figure 3A** indicates that the initial level of circulating bio-ADM relates to the need for and duration of organ support in survivors ($p < 0.0001$).

Concerning circulating bio-ADM levels and cardiovascular function, we found an almost linear relationship of bio-ADM and both cardiovascular SOFA subscore ($p < 0.001$) (**Figure S5**) and duration of cardiovascular drug support (**Figure 3B**) ($p < 0.0001$). Understandably, patients with high bio-ADM needed norepinephrine at admission more frequently (73% versus 42%, $p < 0.0001$) and at greater dose (0.4 [0.3–0.8] versus 0.2 [0.1–0.4] μg/kg/min, $p = 0.0022$) than patients with low bio-ADM (**Table S3**). Our analysis further revealed that patients with high bio-ADM at admission needed more vasopressors/inotropes over the following 7 days even if they did not have those treatments at admission (**Figure 3C**). Regarding other organ support, patients who needed volume resuscitation of more than 5 L over the first 2 days (**Figure 3D**) ($p < 0.0001$) or RRT (**Figure S6**) or had long ICU stay (**Figure S7**) had much higher circulating bio-ADM levels upon ICU admission than those patients who did not.

In agreement with the fact that serial measurements of bio-ADM indicated survival benefit in patients who dropped bio-ADM levels at day 2, we could demonstrate that drop of bio-ADM over the first 2 days also preceded the decrease of total SOFA score (p-value for differences between HH vs. HL: $p < 0.0001$ for all days) (**Figure 4**).

Finally, using the Sepsis-3 definition of septic shock (i.e., vasopressor use and lactate ≥ 2mmol/L [or 18 mg/dl] despite adequate volume resuscitation[17]), our analysis confirmed that bio-ADM upholds a strong prognostication for organ recovery and survival in AdrenOSS-1 (both $p < 0.0001$) (**Figure S8A and B**).

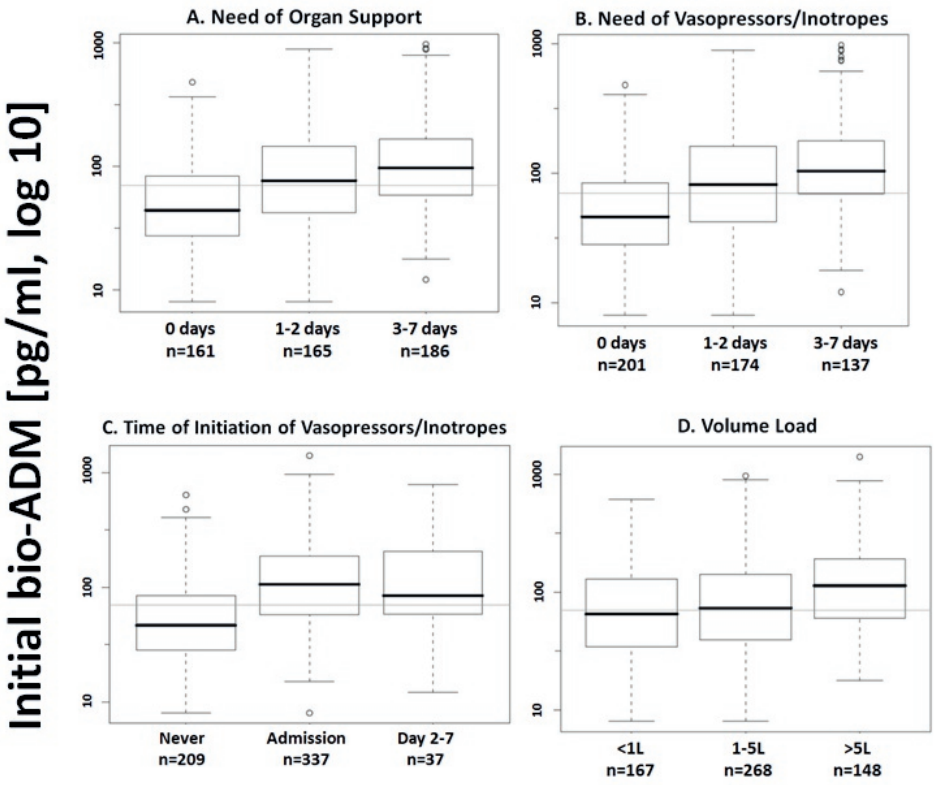

Figure 3. Association between biologically active adrenomedullin levels upon admission and (**A**) length of total organ support over the first 7 days ($p < 0.0001$), (**B**) length of vasopressor/inotropic support over the first 7 days ($p < 0.0001$), (**C**) overall need for vasopressor support ($p < 0.0001$), and (**D**) total fluid balance over the initial 48 h ($p = 0.0001$).

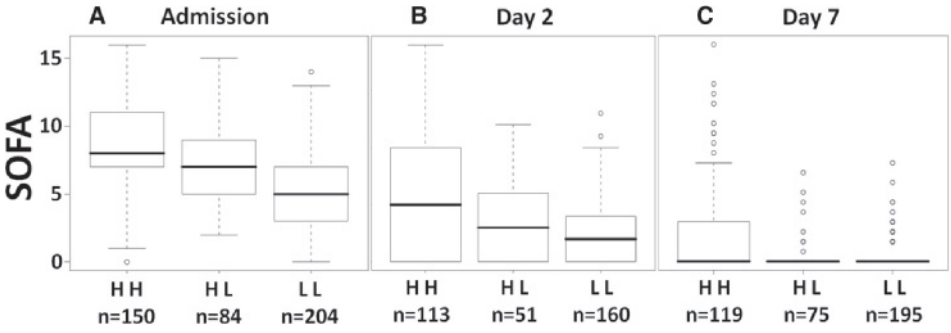

Figure 4. The absolute Sequential Organ Failure Assessment (SOFA) scores at (**A**) admission, (**B**) day 2, and (**C**) day 7 for groups high-high (HH; i.e., above 70 pg/ml at baseline and day 2), high-low (HL), and low-low (LL), excluding patients who died within 7 days. P-value for differences between HH vs. LL: $p < 0.0001$ for all days; p-value for HH vs. HL: $p < 0.0001$ for all days; p-values for HL vs. LL: $p < 0.0001$, 0.6016, and 0.9969 for days 1, 3, and 7, respectively. Of note, the number of patients is less at day 2 than at day 7 because there were more values missing at day 2 owing to the fact that discharged patients (mostly at day 7) were given a SOFA score of 0. Furthermore, only a small number (n = 16, 2.7%) of patients who presented with a low bio-ADM concentration upon admission had a higher bio-ADM level on day 2 (low-high [LH] group), which is why this group is not represented in the figure. Median [IQR] SOFA scores for the LH group were 7.5 [6.0–9.8], 9.0 [4.0–11.2], and 4.0 [0.0–6.5] for admission, day 2, and day 7, respectively.

Discussion

The AdrenOSS-1 study was a prospective multinational observational cohort study assessing the relationship between rapid changes in circulating bio-ADM levels in the first 2 days and clinical outcome in ICU patients with sepsis and septic shock. We confirmed elevated levels of bio-ADM in septic patients and the striking relationship between circulating bio-ADM at ICU admission, organ dysfunction, and death. We also demonstrated that early recovery of circulating bio-ADM levels towards normal values (i.e. <70 pg/ml) was associated with normalization of vascular function and better 28-day survival.

Our study found moderately elevated circulating levels of bio-ADM at admission in sepsis and strongly elevated bio-ADM levels in patients with septic shock, in accordance with earlier reports[13,14]. Our study also confirmed the marked association between bio-ADM level at admission and short-term mortality as well as the prognostic cutoff value of 70 pg/ml, previously described by Marino et al.[14] and Caironi et al.[13] in both sepsis and septic shock (including the most recent definition[17]). Our study showed moderate prognostic value of bio-ADM at admission using AUC but marked prognostic value using Cox proportional hazards model adjusted for various parameters. Moreover, our study showed that prognostic value of bio-ADM at ICU admission exerts additive value (positive changes in chi-square) to various ICU severity scores. We described also the association between a bio-ADM ≤70 pg/ml on day 2 and very low 28-day mortality, even in patients with initial high bio-ADM levels. The association of low bio-ADM by day 2 with full restoration of organ function at day 7 has been shown as well.

Concerning organ dysfunction, we found a relationship between circulating bio-ADM at ICU admission and the subsequent need for cardiovascular and/or renal support. In our studied patients, high circulating bio-ADM—known to have vasodilatory actions—might account for the deterioration of vascular tone and blood pressure, as previously described[13,14]. In the present study, patients with high bio-ADM levels on ICU admission were more likely to need vasopressors and/or inotropes either at admission or in the following days. Moreover, they had a higher total fluid balance and higher incidence of RRT during their ICU stay. The ADM-induced vascular dysfunction may have contributed to this condition, although some data suggest that high bio-AM levels might also be protective to the kidney[19,20]. Further studies are

needed to elucidate the exact role of bio-ADM in renal function. Of interest, the relationship between circulating bio-ADM levels and extent of organ dysfunction, present during ICU admission, was also true during the recovery phase. Indeed, bio-ADM levels decreased before the improvement of total SOFA score in our investigation. Patients with high bio-ADM levels at ICU admission who showed a decline towards normal bio-ADM values at day 2 were more likely to recover vascular function and vasopressor need by day 7. By contrast, the drop in bio-ADM from ICU admission to day 2 was associated with only limited improvement in renal function or no improvement in lung function at day 7. These observations also warrant further exploration.

Circulating bio-ADM levels were lower in AdrenOSS-1 than in the previously described ALBIOS cohort[13]. Indeed, in ALBIOS, septic patients were more severe, as suggested by greater prevalence of mechanical ventilation, length of stay, and short-term mortality. Likewise, the prevalence of septic shock was greater in ALBIOS than in AdrenOSS-1 (**Table S4**). Of note, different definitions of septic shock in the two studies may have influenced study assessments.

Limitations included that in the present population only patients with sepsis and septic shock were studied, and results cannot be directly translated to a general ICU population. Future studies should focus on extrapolation of our results to patients with hemodynamic instability related to other disease, because as study has already been performed for cardiogenic shock[21]. Furthermore, our data suggest that ADM may be associated with myocardial function (e.g. patients with high ADM also had significantly higher circulating natriuretic peptide levels). However, data on cardiac function (e.g. cardiac output or left ventricular ejection fraction) were available in only few studied patients. Finally, we used the cut point of 70 pg/ml of circulating bio-ADM for validation of the previously published cut point, even though the optimal Youden cut points in AdrenOSS-1 showed that 70 pg/ml with respect to a technical optimality criterion is not optimal.

Strong points of the study are the fact that it was a prospective international multicenter study with a large number of patients, with a focus on mortality and organ dysfunction. However, as is true of any observational study, only associations can be described, and cause-and-effect relationships cannot be deducted.

Conclusion

In this large prospective international cohort of critically ill patients admitted to the ICU with sepsis or septic shock, we confirmed the strict relationship between high levels of bio-ADM at ICU admission and organ dysfunction and mortality. We demonstrated that early decrease towards the normal values of circulating bio-ADM in the first days after ICU admission was associated with improvement of cardiovascular and renal function and was associated with very low 28-day mortality.

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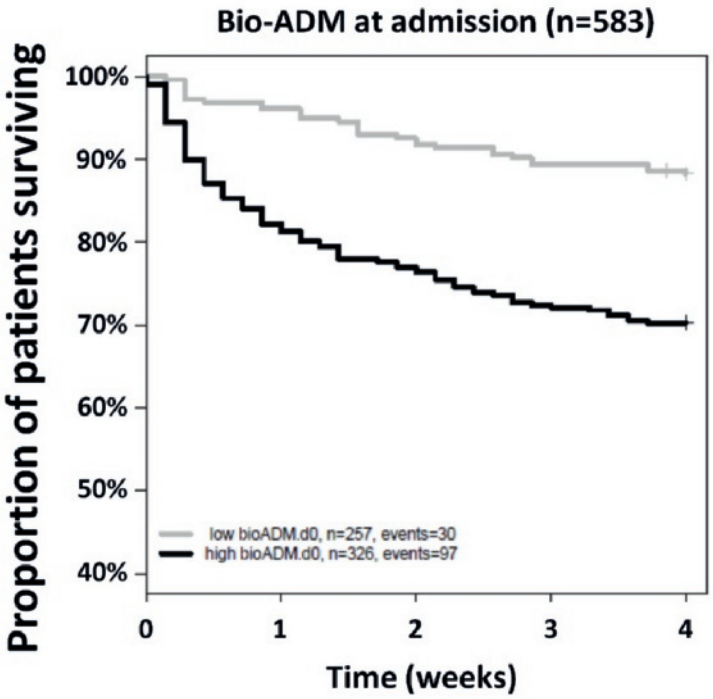

Figure S1. Twenty-eight-day Kaplan-Meier survival curves of low versus high bio-ADM at admission (bioADM.d0) in all patients, based on a cutoff value of 70 pg/ml.

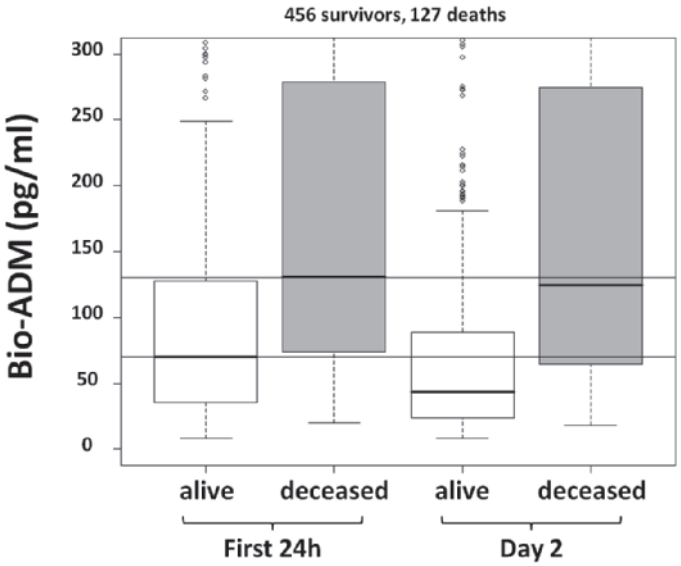

Figure S2. Bio-ADM levels at baseline and on day 2 in 28-day survivors and nonsurvivors. If data were missing at day 2 (e.g., owing to death or discharge; 12.7%), the last available measurement was carried forward. Horizontal lines at 70 and 130 pg/ml for better orientation; y-axis is truncated at 300 pg/ml.

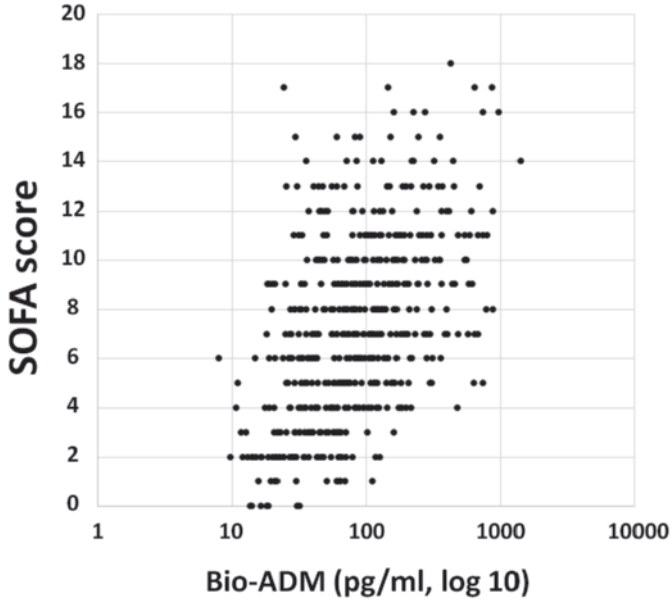

Figure S3. Association between the initial bio-ADM concentration and initial SOFA score ($r = 0.49$, $n = 509$, $p < 0.0001$; missing values due to missing SOFA score components).

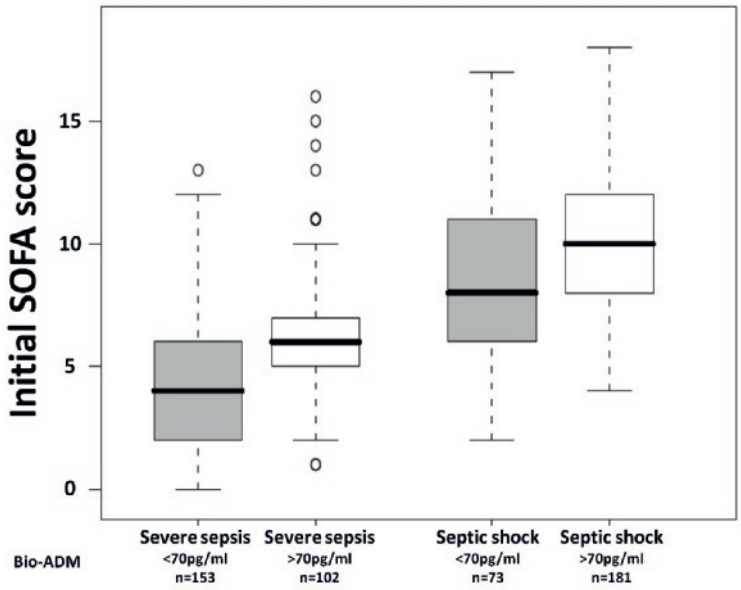

Figure S4. Association of initial SOFA score by sepsis and septic shock and initial bio-ADM concentration below or above 70 pg/ml ($p < 0.0001$ for both bio-ADM and diagnosis; $p = 0.2015$ for interaction; two-way analysis of variance). All data are from admission.

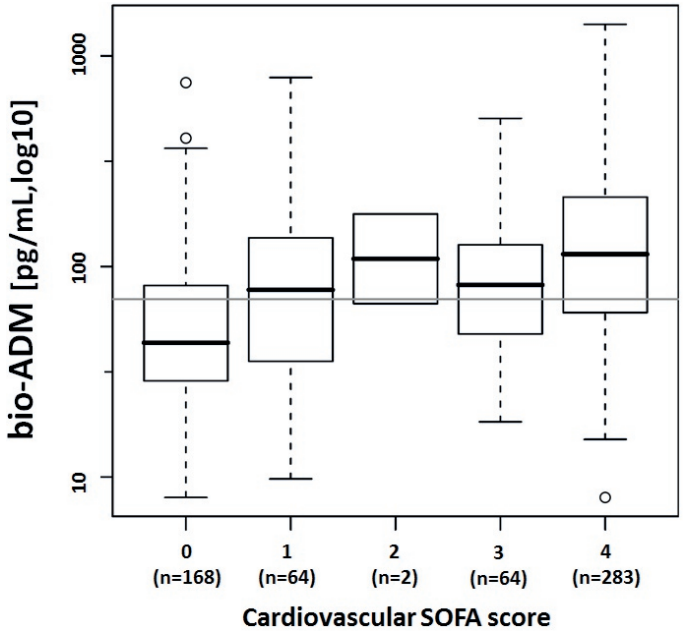

Figure S5. Relationship between bio-ADM and cardiovascular SOFA subscore ($p < 0.001$).

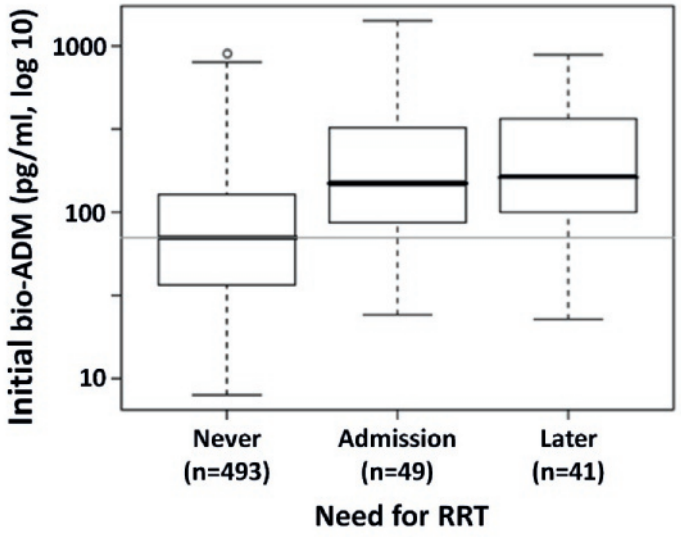

Figure S6. Association between bio-ADM concentration on admission and need for renal replacement therapy on admission, later during ICU stay, or never (70.4 [36.3–128.8] vs. 149.0 [87.1–320.5] and 162.6 [99.8–367.3] pg/ml, for patients without need for RRT, on admission, or later during ICU stay, respectively, $p < 0.0001$).

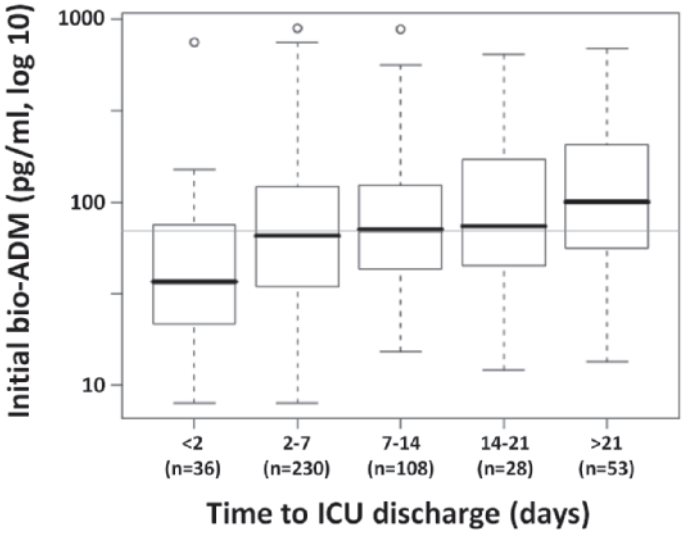

Figure S7. Bio-ADM levels upon admission in 28-day survivors and time to ICU discharge ($p < 0.0001$): Patients with early discharge (< 2 days) are significantly different from all other groups (all $p < 0.016$), and late discharge (> 21 days) is significantly different from early discharge (< 2 days and 2–7 days, both $p < 0.013$).

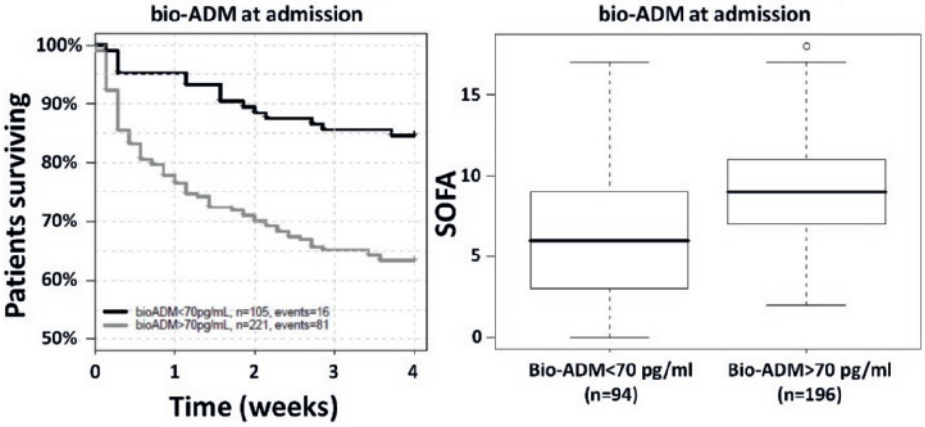

Figure S8. (A) Twenty-eight-day Kaplan-Meier survival curves of low versus high bio-ADM at admission, based on a cutoff value of 70 pg/ml, in patients with lactate > 2 mmol/L ($p < 0.0001$) and (**B**) SOFA score for low versus high bio-ADM at admission ($p < 0.0001$).

Table S1. Patient characteristics of survivors and non-survivors.

Patient characteristics	all	28-day survivors	28-day non-survivor	p-value*	n
Epidemiological data	n=583	n=456	n=127		
Bio-ADM at admission (pg/ml)	80.5 [41.5-148.0]	70.2 [35.8-127.4]	131.0 [74.0-278.5]	<0.0001	
Age (year)	66 [55-76]	65 [54-74]	70 [62-78]	0.0001	
Males (n, %)	364 (62.4)	278 (61)	86 (67.7)	0.1985	
Body Mass Index (kg/m²)	25.7 [22.9-30.1]	25.75 [22.5-30.3]	25.52 [23.7-29.8]	0.5693	
Septic shock at admission	293 (50.3)	199 (43.6)	94 (74)	<0.0001	
Type of ICU admission (%):				0.5724	
Medical	473 (81.1)	374 (82)	99 (78)		
Surgical - emergency procedure	93 (16)	69 (15.1)	24 (18.9)		
Surgical - elective procedure	17 (2.9)	13 (2.9)	4 (3.1)		
Origin of sepsis (%):				0.0408	
Lung	218 (37.4)	176 (38.6)	42 (33.1)		
Blood stream	90 (15.4)	81 (17.8)	9 (7.1)		
Urinary tract	62 (10.6)	43 (9.4)	19 (15)		
Catheter	29 (5)	23 (5)	6 (4.7)		
Peritonitis	31 (5.3)	24 (5.3)	7 (5.5)		
Endocarditis	31 (5.3)	21 (4.6)	10 (7.9)		
Bile duct infection	8 (1.4)	6 (1.3)	2 (1.6)		
CNS	4 (0.7)	3 (0.7)	1 (0.8)		
Skin and soft tissue	10 (1.7)	9 (2)	1 (0.8)		
Gynaecologic	2 (0.3)	2 (0.4)	0 (0)		
Other	98 (16.8)	68 (14.9)	30 (23.6)		
Medical history** (%)					
Any cardiac comorbidity	400 (68.6)	298 (65.4)	102 (80.3)	0.0019	
Chronic Heart Failure	60 (10.3)	43 (9.4)	17 (13.4)	0.2530	
Hypertension	293 (50.3)	214 (46.9)	79 (62.2)	0.0019	
Diabetes mellitus	160 (27.4)	120 (26.3)	40 (31.5)	0.2793	
Any non-cardiac comorbidity	414 (71)	309 (67.8)	105 (82.7)	0.0015	
Chronic renal disease	76 (13.0)	57 (12.5)	19 (15.0)	0.5586	
Active/recent malignant tumors	124 (21.3)	88 (19.3)	36 (28.3)	0.0272	
Smoking (active)	117 (20.1)	90 (19.7)	27 (21.3)	0.8099	
COPD	89 (15.3)	67 (14.7)	22 (17.3)	0.4932	
Any chronic medication	371 (63.6)	278 (61)	93 (73.2)	0.0148	
Immunosuppressive therapy	46 (7.9)	37 (8.1)	9 (7.1)	0.8464	
Physiological values at admission					
Temperature (°C)	37.2 [36.4-38.2]	37.3 [36.6-38.2]	36.8 [36-37.8]	0.0010	
Mean blood pressure (mmHg)	75 [64-90]	76 [65-91]	73 [60-90]	0.1322	
Heart rate (bpm)	104 [90-119]	103 [88-117]	110 [94-130]	0.0077	
Central Venous pressure (mmHg)	8 [5-13]	8 [5-13]	10 [6-13]	0.4690	
Glasgow score	15 [14-15]	15 [14-15]	15 [12-15]	<0.0001	
Fluid Balance (ml)	1928 [592-3552]	1745 [500-3131]	2853.5 [1145-5036]	<0.0001	
Urine output for 24 hours (ml)	1000 [450-1900]	1158 [583-1996]	550 [223-1440]	<0.0001	
PaO_2/FiO_2	228 [137-340]	234 [146-354]	190 [114-298]	0.0059	

Table S1. continued.

Patient characteristics	all	28-day survivors	28-day non-survivor	p-value*	n
Laboratory values at admission					
Lactate (mmol/l)	1.4 [1.0-2.2]	1.2 [0.9-1.9]	2.2 [1.4-4.0]	<0.0001	n=562
Arterial pH	7.38 [7.3-7.44]	7.4 [7.32-7.45]	7.33 [7.27-7.4]	<0.0001	
Bilirubin (umol/L)	11 [6-19]	10 [6-18]	13 [7-24]	0.0961	
Platelets (10^9/L)	190 [121-275]	193 [124-281]	166 [96-261]	0.0301	
Creatinine (mg/dL)	1.4 [0.9-2.2]	1.3 [0.8-2.1]	1.6 [1.1-2.6]	0.0023	
BUN or Urea (mg/dL)	61 [37-107]	57 [34-101]	75 [49-120]	0.0002	
Hematocrit (%)	34 [29-38]	34 [30-38]	33 [28-39]	0.4325	
White blood count (per mm^3)	12525 [7200-18585]	13345 [8000-18975]	10365 [4150-16770]	0.0008	
Troponin T, maximum on day 1	42 [18-158]	33 [18-131]	61 [34-241]	0.0989	n=153
Troponin I, maximum on day 1	69 [20-246]	60 [17-228]	150 [39-1001]	0.0468	n=186
PCT, maximum on day 1 (ng/mL)	11.4 [1.9-49.8]	10.7 [1.9-49.4]	11.8 [2.2-52.5]	0.6690	n=330
PCT, central lab (ng/mL)	10.2 [2.3-34.3]	9.7 [2.2-31.5]	14.8 [3.9-42.8]	0.0442	n=583
BNP, maximum on day 1	257 [102-723]	239 [87.7-650]	514 [139-1242]	0.1183	n=131
NT-proBNP, maximum on day 1	4382 [1525-11565]	3400.5 [1026.88-9052]	7249 [4564-25007]	0.0007	n=117
Organ support at admission (%)					
Mechanical ventilation:				<0.0001	
Invasive	219 (37.6)	141 (30.9)	78 (61.4)		
Non-invasive	131 (22.5)	107 (23.5)	24 (18.9)		
None	233 (40.0)	208 (45.6)	25 (19.7)		
Renal replacement therapy	49 (8.4)	27 (5.9)	22 (17.3)	0.0002	
Vasopressors/inotropes at admission	346 (59.9)	246 (53.9)	103 (81.1)	<0.0001	
Organ dysfunction scores					
SOFA (points)	7 [5-10]	6 [4-9]	10 [7.5-12]	<0.0001	n=509
APACHE II (points)	15 [11-20]	15 [10-19]	19 [16-23.5]	<0.0001	
Length of stay (days)					
ICU	5 [2-10]	5 [2-10]	4 [2-8]	0.0147	
Mortality					
28-day, deaths (%)	127 (21.8)	0 (0)	127 (100)	<0.0001	
90-day, deaths (%)	166 (28.5)	39 (8.6)	127 (100)	<0.0001	

* p-value from non-parametric Kruskal-Wallis or Chi^2 test, respectively.
** most common comorbidities reported individually.
Abbreviations: APACHE, acute physiology and chronic health evaluation; Bio-ADM, bioactive adrenomedullin; BNP, brain-derived natriuretic peptide; BUN, blood urea nitrogen; CNS, central nervous system; COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; PCT, procalcitonin; SOFA, sequential organ failure assessment; NT-proBNP, N-terminal brain natriuretic peptide.

Table S2. Patient characteristics of the four different groups with respect to adrenomedullin trajectory over the first 48 h after study inclusion.

Patient characteristics	all	Bio-ADM <70 pg/mL, admission and 48h	Bio-ADM <70 pg/mL on admission, but >70 pg/mL at 48h	Bio-ADM >70 pg/mL on admission, but <70 pg/mL at 48h	Bio-ADM >70 pg/mL, admission and 48h	p-value*	n
Epidemiological data	n=583	n=241 (41.3%)	n=16 (2.7%)	n=95 (16.3%)	n=231 (39.6%)		
Bio-ADM at admission (pg/ml)	80.5 [41.5-148.0]	35.7 [27.0-48.1]	60.3 [55.8-66.0]	95.4 [82.8-120.6]	162.6 [118.0-301.0]	<0.0001	
Age (year)	66 [55-76]	64 [53-75]	64 [55-70]	67 [59-77]	68 [58-76]	0.0454	
Males (n, %)	364 (62.4)	159 (66)	12 (75)	60 (63.2)	133 (57.6)	0.1942	
Body Mass Index (kg/m^2)	25.7 [22.9-30.1]	25.0 [22.5-28.4]	25.1 [21.2-27.9]	25.9 [22.5-30.7]	26.9 [23.4-32.0]	0.0048	
Septic shock at admission	293 (50.3)	75 (31.1)	9 (56.2)	52 (54.7)	157 (68)	<0.0001	
Type of ICU admission (%):						0.0007	
Medical	473 (81.1)	216 (89.6)	14 (87.5)	69 (72.6)	174 (75.3)		
Surgical - emergency procedure	93 (16)	20 (8.3)	1 (6.2)	22 (23.2)	50 (21.6)		
Surgical - elective procedure	17 (2.9)	5 (2.1)	1 (6.2)	4 (4.2)	7 (3)		
Origin of sepsis(%):						<0.0001	
Lung	218 (37.4)	123 (51)	6 (37.5)	30 (31.6)	59 (25.5)		
Blood stream	90 (15.4)	31 (12.9)	0 (0)	23 (24.2)	36 (15.6)		
Urinary tract	62 (10.6)	8 (3.3)	2 (12.5)	13 (13.7)	39 (16.9)		
Catheter	29 (5)	9 (3.7)	0 (0)	8 (8.4)	12 (5.2)		
Peritonitis	31 (5.3)	11 (4.6)	1 (6.2)	2 (2.1)	17 (7.4)		
Endocarditis	31 (5.3)	11 (4.6)	1 (6.2)	6 (6.3)	13 (5.6)		
Bile duct infection	8 (1.4)	2 (0.8)	0 (0)	2 (2.1)	4 (1.7)		
CNS	4 (0.7)	4 (1.7)	0 (0)	0 (0)	0 (0)		
Skin and soft tissue	10 (1.7)	9 (3.7)	0 (0)	1 (1.1)	0 (0)		
Gynaecologic	2 (0.3)	1 (0.4)	0 (0)	0 (0)	1 (0.4)		
Other	98 (16.8)	32 (13.3)	6 (37.5)	10 (10.5)	50 (21.6)		
Medical history**							
Any cardiac comorbidity	400 (68.6)	135 (56)	12 (75)	71 (74.7)	182 (78.8)	<0.0001	
Chronic Heart Failure	60 (10.3)	16 (6.6)	3 (18.8)	10 (10.5)	31 (13.4)	0.0697	
Hypertension	293 (50.3)	97 (40.2)	8 (50.0)	53 (55.8)	135 (58.4)	0.0004	
Diabetes mellitus	160 (27.4)	52 (21.6)	5 (31.2)	22 (23.2)	81 (35.1)	0.0075	

Table S2. continued.

Patient characteristics	all	Bio-ADM <70 pg/mL, admission and 48h	Bio-ADM <70 pg/mL on admission, but >70 pg/mL at 48h	Bio-ADM >70 pg/mL on admission, but <70 pg/mL at 48h	Bio-ADM >70 pg/mL, admission and 48h	p-value*	n
Any non-cardiac comorbidity	414 (71)	153 (63.5)	14 (87.5)	70 (73.7)	177 (76.6)	0.0056	
Chronic renal disease	76 (13.0)	18 (7.5)	1 (6.2)	13 (13.7)	44 (19.0)	0.0019	
Active/recent malignant tumor	124 (21.3)	28 (11.6)	6 (37.5)	26 (27.4)	64 (27.7)	<0.0001	
Smoking (active)	117 (20.1)	60 (24.9)	3 (18.8)	15 (15.8)	39 (16.9)	0.1286	
COPD	89 (15.3)	34 (14.1)	3 (18.8)	19 (20.0)	33 (14.3)	0.5181	
Any chronic medication	371 (63.6)	126 (52.3)	12 (75)	62 (65.3)	171 (74)	<0.0001	
Immunosuppressive therapy	46 (7.9)	10 (4.1)	1 (6.2)	7 (7.4)	28 (12.1)	0.0153	
Physiological values at admission							
Temperature (°C)	37.2 [36.4-38.2]	37.3 [36.6-38.3]	37.65 [36.8-38]	37.1 [36.3-38]	37.1 [36.2-38.1]	0.0320	
Mean blood pressure (mmHg)	75 [64-90]	81 [69-97]	77 [70-84]	73 [61-87]	71 [60-85]	<0.0001	
Heart rate (bpm)	104 [90-119]	99 [86-115]	106 [92-127]	104 [95-121]	107 [94-122]	0.0086	
Central Venous pressure (mmHg)	8 [5-13]	7.5 [5-13]	9 [8-11]	11 [6-14]	10 [6-13]	0.6906	
Glasgow score	15 [14-15]	15 [14-15]	15 [15-15]	15 [14-15]	15 [14-15]	0.2926	
Fluid Balance (ml)	1928 [592-3552]	1366 [483-2673]	1973 [625-2821]	1930 [730-3196]	2629 [813-4798]	0.0000	
Urine output for 24 h (ml)	1000 [450-1900]	1300 [700-2110]	540 [285-1105]	1095 [581-1947]	630 [237-1540]	<0.0001	
PaO_2/FiO_2	228 [137-340]	235 [143-368]	204 [107-269]	237 [155-365]	209 [133-330]	0.3173	
Laboratory values at admission							
Lactate (mmol/l)	1.4 [1.0-2.2]	1.1 [0.8-1.6]	1.3 [1.0-2.2]	1.4 [1.0-2.0]	2.0 [1.2-3.1]	<0.0001	n=562
Arterial pH	7.38 [7.3-7.44]	7.42 [7.36-7.46]	7.38 [7.32-7.44]	7.37 [7.32-7.44]	7.35 [7.26-7.41]	<0.0001	
Bilirubin (umol/L)	11 [6-19]	10 [6-17]	10.5 [7-17]	11.5 [6-22]	12 [6-21]	0.5267	
Platelets (10^9/L)	190 [121-275]	196 [136-275]	191.5 [137-328]	186 [116-262]	178 [103-280]	0.2895	
Creatinine (mg/dL)	1.4 [0.9-2.2]	1 [0.7-1.4]	1.1 [0.8-1.8]	1.8 [1.2-2.6]	1.8 [1.2-3]	<0.0001	
BUN or Urea (mg/dL)	61 [37-107]	43 [28-67]	52 [33-90]	72 [46-121]	82 [53-129]	<0.0001	
Hematocrit (%)	34 [29-38]	35 [30-38]	33.5 [31.5-35.75]	35 [30-38]	33 [28-38]	0.1831	
White blood count (per mm^3)	12525 [7200-18585]	13210 [8507-17932]	11995 [7457-20062]	12375 [7692-18825]	11445 [5427-19075]	0.1783	
Troponin T, max on day 1	42 [18-158]	27 [14-64]	129 [33-554]	37 [23-145]	69 [26-187]	0.0082	n=153
Troponin I, max on day 1	69 [20-246]	45 [11-228]	30 [25-762]	91 [40-330]	100 [40-254]	0.0439	n=186

Table S2. continued.

Patient characteristics	all	Bio-ADM <70 pg/mL, admission and 48h	Bio-ADM <70 pg/mL on admission, but >70 pg/mL at 48h	Bio-ADM >70 pg/mL on admission, but <70 pg/mL at 48h	Bio-ADM >70 pg/mL, admission and 48h	p-value*	n
PCT, max on day 1 (ng/mL)	11.4 [1.9-49.8]	3.8 [0.9-19.7]	4.6 [0.9-6.5]	25.9 [7.7-70.7]	19.3 [4.5-87]	<0.0001	n=330
PCT, max on day 1 (ng/mL)	10.2 [2.3-34.3	3.8 [0.8-13.0]	2.6 [1.3-10.1]	17.4 [5.0-50.9]	18.8 [6.3-53.9]	<0.0001	n=583
BNP, max on day 1	257 [102-723	185 [59-383]	806 [428-592]	372 [140-503]	545 [166-1282]	0.0007	n=131
NT-proBNP, max on day 1	4382 [1525-11565]	2072 [459-5726]	8691 [2301-17225]	4970 [1951-11896]	6229 [3560-18486]	0.0001	n=117
Organ support at admission (%)							
Mechanical ventilation:						0.0004	
Invasive	219 (37.6)	76 (31.5)	9 (56.2)	24 (25.3)	110 (47.6)		
Non-invasive	131 (22.5)	63 (26.1)	4 (25)	22 (23.2)	42 (18.2)		
None	233 (40.0)	102 (42.3)	3 (18.8)	49 (51.6)	79 (34.2)		
Renal replacement therapy	49 (8.4)	8 (3.3)	0 (0)	7 (7.4)	34 (14.7)	0.0008	
Vasopressors/inotropes at admission (%)	349 (59.9)	98 (40.7)	11 (68.8)	59 (62.1)	181 (78.4)	<0.0001	
Organ dysfunction scores							
SOFA (points)	7 [5-10]	5 [3-8]	7.5 [6-10]	7 [5-9]	9 [7-11]	<0.0001	n=509
APACHE II (points)	15 [11-20]	14 [9-17]	15.5 [13-19.5]	16 [12.5-20]	18 [14-22.5]	<0.0001	
Length of stay (days)							
ICU	5 [2-10]	4 [2-8]	10 [7.5-15.5]	5 [3-7.5]	5 [2-13]	0.0002	
Mortality							
28-day, deaths (%)	127 (21.8)	25 (10.4)	5 (31.2)	9 (9.5)	88 (38.1)	<0.0001	
90-day, deaths (%)	166 (28.5)	36 (14.9)	5 (31.2)	16 (16.8)	109 (47.2)	<0.0001	

* p-value from non-parametric Kruskal-Wallis or Chi2 test, respectively.
** most common comorbidities reported individually.
Abbreviations: APACHE, acute physiology and chronic health evaluation; Bio-ADM, bioactive adrenomedullin; BNP, brain-derived natriuretic peptide; BUN, blood urea nitrogen; CNS, central nervous system; COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; PCT, procalcitonin; SOFA, sequential organ failure assessment; NT-proBNP, N-terminal brain natriuretic peptide.

Table S3. Association between ADM and need of vasopressors/inotropes at admission.

	All	Bio-ADM <70 pg/mL	Bio-ADM >70 pg/mL	p-value
Dobutamine (%)	23 (4)	9 (4)	14 (4)	0.7842
Dobutamine (%)	23 (4)	9 (4)	14 (4)	0.7842
Epinephrine (%)	16 (3)	3 (1)	13 (4)	0.0696
Norephinephrine (%)	346 (59)	107 (42)	239 (73)	<0.0001
Dose of norepinephrine (mg/kg/min; median [IQR])	0.3 [0.2-0.7]	0.2 [0.1-0.4]	0.4 [0.3-0.8]	0.0022

Data are expressed as median [IQR, interquartile range] or as number of patients (percentage). *Abbreviations: Bio-ADM, bioactive adrenomedullin.*

Table S4. Comparison of AdrenOSS-1 and ALBIOS.

	AdrenOSS-1 n = 583	ALBIOS n = 956
Definition of septic shock	Sepsis-2	SOFA score 3-4
Septic shock patients (%)	50.3	56.4
Baseline Bio-ADM value (median, pg/mL)	80.5	110
Bio-ADM value in severe sepsis (median, pg/mL)	58	86
Bio-ADM value in septic shock (median, pg/mL)	114	122
Mechanical ventilation upon study inclusion (%)	60	82.5
Length of ICU stay (median, IQR)	5 [2-10]	10 [5-20]
90-day mortality (%)	29	39

Data are expressed as time range, median [IQR, interquartile range] or as number of patients (percentage). *Abbreviations: Bio-ADM, bioactive adrenomedullin.*

Part III

Preclinical evaluation of
the adrenomedullin-binding antibody
Adrecizumab

Chapter 9

Effects of the humanized anti-adrenomedullin antibody Adrecizumab (HAM8101) on vascular barrier function and survival in rodent models of systemic inflammation and sepsis

Christopher Geven, Esther Peters, Mathias Schroedter, Joachim Struck, Andreas Bergmann, Oscar McCook, Peter Radermacher, Matthijs Kox and Peter Pickkers.

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Abstract

Purpose

Adrenomedullin (ADM) is an important regulator of endothelial barrier function during sepsis. Administration of a murine antibody targeted against the N-terminus of ADM (HAM1101) resulted in improved outcome in models of murine sepsis. We studied the effects of a humanized form of this antibody (HAM8101, also known as Adrecizumab) on vascular barrier dysfunction and survival in rodent models of systemic inflammation and sepsis.

Methods

Rats (n=48) received different dosages of HAM8101 or placebo (n=8 per group), directly followed by administration of lipopolysaccharide (5 mg/kg). Twenty-four hours later, Evans Blue dye was administered to assess vascular leakage in kidney and liver tissue. Furthermore, mice (n=24) were administered different dosages of HAM8101 or placebo (n=6 per group), immediately followed by cecal ligation and puncture (CLP). Eighteen hours later, albumin, vascular endothelial growth factor (VEGF), and angiopoietin-1 were analyzed in the kidney. Finally, effects of single and repeated dose administration of HAM1101, HAM8101 and placebo on survival were assessed in CLP-induced murine sepsis (n=60, n=10 per group).

Results

Dosages of 0.1 and 2.5 mg/kg HAM8101 attenuated renal albumin leakage in endotoxemic rats. Dosages of 0.1, 2.0, and 20 mg/kg HAM8101 reduced renal concentrations of albumin and the detrimental protein VEGF in septic mice, whereas concentrations of the protective protein angiopoietin-1 were augmented. Both single and repeated administration of both HAM1101 and HAM8101 resulted in improved survival during murine sepsis.

Conclusions

Pretreatment with the humanized anti-ADM antibody HAM8101 improved vascular barrier function and survival in rodent models of systemic inflammation and sepsis.

Background

Sepsis is an inflammatory syndrome, in which a dysregulated host response to an infection results in life-threatening organ dysfunction[1]. It is a frequent reason for admission to the intensive care unit[2], and despite many advances in medical care, its incidence is increasing[3] and mortality remains high[4]. The current treatment of sepsis consists of source control, antimicrobial therapy, and supportive treatment such as fluid resuscitation, vasopressor use, and mechanical ventilation[5]. Currently, no adjunctive pharmacological therapies are used in clinical practice[6]. Hemodynamic instability plays an important role in the development of septic shock, and arises due to a combination of sepsis-induced vasodilation and vascular leakage, the latter of which is caused by disrupted endothelial integrity. Extensive changes occur in the endothelium as a result of circulating damage-associated molecular patterns and pathogen-associated molecular patterns that activate inflammatory and coagulation pathways during sepsis. In turn, this can result in increased leukocyte adhesion, a procoagulant state, vasodilation and endothelial cell permeability, and ultimately widespread edema, shock and lethal organ dysfunction[5,7,8].

Adrenomedullin (ADM) appears to play an important role in the regulation of the endothelial barrier function and modulation of vascular tone. ADM is a free circulating peptide synthesized by several cell-types, including vascular endothelial and vascular smooth muscle cells[9,10]. ADM signals through binding to the AM1- and AM2-receptors, which are composed of a calcitonin-receptor-like receptor (CLR), and receptor activity modifying proteins (RAMP2 or RAMP3 for AM1 and AM2, respectively)[11]. Results on the role of ADM in sepsis are ambiguous. During sepsis, ADM levels are correlated with relaxation of vascular tone[12] as well as with disease severity and mortality in septic patients[13–15]. In vitro and in vivo data demonstrate that ADM exerts beneficial effects on the endothelial barrier, as it prevents endothelial hyperpermeability and subsequent edema formation by inhibition of actin-myosin-based endothelial cell contraction and junctional disruption[16–19]. Furthermore, ADM administration was shown to improve gut microcirculation in models of inflammation[17,20]. However, other work revealed that ADM promotes vasodilation, and infusion of high doses of ADM decreases blood pressure and induces a compensatory increased heart rate in rats, cats, sheep, and humans[21–25]. These data suggest that the ADM response needs to be tightly regulated, maintaining adequate, but avoiding excessive signaling.

Recently, a high-affinity antibody targeting the N-terminus of ADM (HAM1101), which only partially inhibits ADM signaling, showed beneficial effects on outcome in two cecal ligation and puncture (CLP) studies[26,27]. The observation that the antibody that only partially inhibits ADM exerts more benefit than the antibody that completely blocks ADM[26], supports the paradigm that a moderate ADM response is needed. Subsequently, a humanized version of this antibody (HAM8101, also known as Adrecizumab) has been developed for clinical use. In the present study, we investigated the effects of HAM8101 on vascular barrier dysfunction in a rat model of systemic inflammation induced by endotoxin administration as well as in the more clinically relevant model of sepsis induced by cecal ligation and puncture in mice (CLP)[28]. Furthermore, we compared the effects of HAM8101 and HAM1101 on survival in the murine CLP model.

Methods

Animals

Animal experiments were performed according to the guidelines of the Federation of Laboratory Animal Science Associations (FELASA) and the society of Laboratory Animal Science (GV-SOLAS). All animals were obtained from Charles River Laboratories, Sulzfeld, Germany. Male Wistar rats were used for the lipopolysaccharide (LPS) vascular permeability study (n=48, aged 2–4 months) performed by Preclinics (Potsdam, Germany). The CLP kidney barrier dysfunction study was performed by Phenos GmbH (Hannover, Germany), and male mus musculus C57BL/6 mice (n=24, 12–15 weeks old) were used. For the CLP survival experiments (also performed by Phenos GmbH), 12 to 15-week-old male mus musculus C57BL/6 mice were used (n=30 for single and n=30 for repeated dose experiments respectively. These were separate experiments, using different batches per survival experiment). To determine the robustness of HAM8101 during sepsis/inflammation, its efficacy was investigated in different animal species, using different sepsis models and end-points. Animals were housed under routine laboratory conditions, kept under 12h/12h light–dark cycle conditions and fed ad libitum with standard chow (Ssniff R/M-H diet) with unlimited access to water. Substance administration, surgery, and blood withdrawal were performed under isoflurane anesthesia to avoid stress and pain in animals. This study is reported according to the ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines.

Antibodies

Generation of the murine monoclonal anti-Adrenomedullin antibody HAM1101 has been described previously[26,27]. From this murine monoclonal antibody a humanized recombinant monoclonal antibody (HAM8101;IgG1) was generated by CDR grafting, and it was produced in Chinese hamster ovary cells. HAM8101 was produced by Glycotope Biotechnology GmbH (Heidelberg, Germany) under Good Manufacturing Practice (GMP) conditions, and this antibody is also known as Adrecizumab; HAM1101 was produced by InVivo GmbH (Hennigsdorf, Germany). The antibodies are directed against the N-terminus of ADM. The antibodies were kindly provided by Adrenomed AG (Hennigsdorf, Germany) in stock solutions in PBS and were stored at 2°C to 8°C under temperature controlled and restricted access conditions.

LPS-induced vascular permeability study procedures

Rats (n=48 total) were randomly divided into six groups (n=8 each), of which one group received placebo twice (saline and PBS [control]), whereas the other five groups received 5mg/kg bodyweight LPS (E coli 055:B5; Sigma-Aldrich, Taufkirchen, Germany) in combination with different dosages of HAM8101 (Adrecizumab, 0.02 mg/kg, 0.1 mg/kg, 0.5 mg/kg, or 2.5 mg/kg) or PBS. HAM8101/PBS was administered 5 min before LPS/saline. All substances were administered as a bolus intravenously through a 24G indwelling catheter placed in the tail vein. 300 µL blood from each animal was collected in Multivette K-EDTA-tubes (Sarstedt, Nümbrecht, Germany) through the tail vein at baseline as well as 3, 6, and 24 h after injection of LPS/saline. Tubes were stored on ice until centrifugation (3,220 × g at 6°C for 10 min). Plasma was then transferred to a micro tube and stored at –80°C until analysis of plasma ADM concentrations. Twenty-four hours after LPS/saline administration, deep isoflurane anesthesia was induced and Evans Blue (40mg/kg, dissolved in saline, [Sigma-Aldrich, Taufkirchen, Germany]) was administered. Approximately 15 min after Evans Blue administration, the aorta was cannulated and whole body perfusion was started (15 min, saline + 50 IU/mL heparin [Heparin-Sodium 25000 I.E./5mL; B. Braun Melsungen AG, Melsungen, Germany]). Following perfusion, kidney and liver was harvested and directly processed for Evans Blue absorption (see further below).

CLP-induced kidney barrier dysfunction study procedures

Mice were randomly divided into four groups (n=6 each, n=24 total) and received a single i.v. injection of saline (control group) or HAM8101 (0.1 mg/kg, 2 mg/kg, or 20 mg/kg, respectively), 5 min prior to CLP surgery by tail vein injection. Peritonitis was surgically induced under isoflurane anesthesia. Incisions were made into the left upper quadrant of the peritoneal cavity (normal location of the cecum). The cecum was exposed and a tight ligature was placed around the cecum (at 70–75% cecum length). One puncture wound was made with a 24 G needle into the cecum and small amounts of cecal contents were pressed through the wound. The cecum was returned into the peritoneal cavity and the laparotomy site was closed. Animals were returned to their cages with free access to food and water. To prevent dehydration, 500 µL saline was administered subcutaneously. For analgesic treatment, carprofen (Rimadyl, Zoetis, Berlin, Germany) 5 mg/kg was administered subcutaneously directly after surgery. Eighteen hours following surgery, mice were sacrificed by retrobulbar exsanguination, whereafter the left kidney was removed immediately and cut in two parts, which were processed for immunohistochemistry (see further below).

CLP survival study procedures

In the single-dose administration study, mice (n=30 total) were randomly divided into three groups (n=10 each) to receive either a single dose of PBS, 2 mg/kg HAM1101, or 2 mg/kg HAM8101, administered by intravenous tail vein injection, 5 min prior to CLP surgery (performed as described in the previous section "CLP-induced kidney barrier dysfunction study procedures"). For analgetic treatment, 5mg/kg carprofen (Rimadyl, Zoetis, Berlin, Germany) was injected subcutaneously after surgery, and metamizole (Novalgin, Sanofi-Aventis, Frankfurt, Germany) was added to the drinking water for 3 days after CLP surgery (0.8 mL / 500 mL water). To prevent dehydration, 500 µL saline was administered subcutaneously. Mice were followed up for 7 days and physical condition was monitored twice daily. If still alive on day 7, animals were sacrificed by retrobulbar exsanguination. In the repeated dose administration study, mice (n=30 total) were randomly divided into three groups (n=10 each) to receive doses of either PBS, 4 mg/kg HAM1101 or 4 mg/kg HAM8101 5 min prior to CLP surgery (performed as described in the previous section "CLP-induced kidney barrier dysfunction study procedures"). To prevent dehydration, 500 µL saline was administered subcutaneously. Additional dosages of PBS, 2 mg/kg HAM1101, or 2 mg/kg

HAM8101 were administered 24 and 48h after CLP surgery by retrobulbar injection. Mice were followed up for 14 days, in which physical conditions were monitored twice daily, and if still alive on day 14, they were sacrificed by retrobulbar exsanguination.

Evans Blue analysis

Organs were homogenized and incubated in 1mL formamide per gram organ sample (Sigma-Aldrich, Taufkirchen, Germany) at 55°C for 3h to extract Evans Blue. Subsequently, the solution was transferred to fresh centrifugation tubes, of which 500 µL was transferred onto a centrifugal filter (3K, VWR, Radnor, Pa) unit and centrifuged at 25,000 × g at room temperature for 30min. The filtrate was transferred to a 96-well-plate (non-treated; Costar) and absorption of samples and reference standards was measured at 620nm using a plate reader (Mithras LB940; Berthold Technologies, Wildbad, Germany).

Immunohistochemistry

The left kidney tissues were formalin-fixed, dehydrated, and embedded in paraffin. Study procedures were done as described previously[29]. In short, 5-µm-thick kidney sections were cut and deparaffinized with xylene, graded ethanol, and deionized water, followed by heat-induced antigen retrieval performed by microwaving with citrate buffer. Depending on the source of the secondary antibody, blocking was performed with normal (goat or donkey) serum. Next, primary antibodies were used for immunohistochemical localization of VEGF (rabbit polyclonal, Abcam, Cambridge, UK), angiopoietin-1 (goat polyclonal, R&D Systems, Minneapolis, Minn), and extravasation of albumin (goat polyclonal M-13, Santa Cruz Biotech, Santa Cruz, Calif). The primary antibodies were detected with secondary antibodies (goat anti-rabbit or donkey anti-goat IgG alkaline phosphatase), followed by Dako REAL Detection System Chromogen Red, and counterstained with Mayer's hematoxylin. Slides were analyzed using the Axio Vision software (release 4.8), multiple 800,000 µm2 fields were evaluated, and data are represented as mean densitometric sums red.

Adrenomedullin assay

The ADM assay was performed as described previously[13]. Briefly, plasma ADM (both free and antibody-bound) concentrations were measured using a sandwich-coated luminescence immunoassay, based on acridinium NHS-

ester labeling and anti-ADM antibodies (a solid phase antibody targeted against the mid region of ADM and a labeled antibody against the amidated C-terminal moiety of ADM). Dilutions of rat ADM and labeled tracer were subsequently added to antibody-coated wells. After washing of unbound tracer, chemiluminescence was measured and evaluated against a standard curve from rat ADM standards.

Statistical analysis

Data were non-normally distributed, and therefore expressed as median and interquartile range. Differences across three or more groups were analyzed using Kruskal–Wallis tests. In case the two-sided P-value of the Kruskal–Wallis test was < 0.05, pairwise comparisons were made using Mann–Whitney U tests. P-values of the Mann–Whitney U tests were not adjusted for multiple testing in view of the exploratory nature of the experiments. For survival analysis, Kaplan–Meier curves were generated and log-rank tests were performed to test statistical significance of observed differences. A two-sided P-value < 0.05 was considered statistically significant. Calculations and statistical analyses were performed using GraphPad Prism version 6 for Windows (Graphpad Software Inc, La Jolla, California, USA).

Results

HAM8101 prevents LPS-induced vascular hyperpermeability in the kidney

The effect of HAM8101 on LPS-induced vascular hyperpermeability was studied in kidney and liver tissue using Evans Blue dye to detect albumin leakage. LPS administration resulted in a 3.5-fold increase in renal albumin leakage compared with saline-treated controls, which was significantly attenuated by HAM8101 at dosages of 0.1 mg/kg and 2.5 mg/kg (71% and 40% attenuation, respectively), whereas statistical significance was not reached for the 0.5 mg/kg dose (33% attenuation, P=0.083, **Figure 1A**). LPS administration also resulted in a 2.3-fold increase in hepatic albumin leakage (**Figure 1B**). Only the highest dose of HAM8101 tended to attenuate albumin leakage in the liver, approaching but not reaching statistical significance (36% attenuation, P=0.065, **Figure 1B**). LPS administration resulted in increased ADM plasma levels (**Figure 2**). HAM8101 administration in dosages of 0.5 mg/kg or higher caused a dose-dependent increase in plasma ADM concentrations (**Figure 2**).

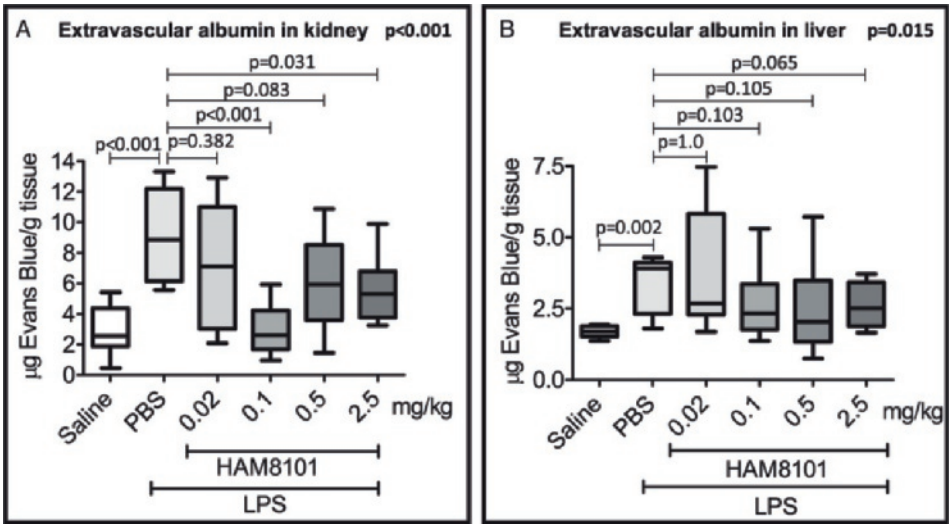

Figure 1. Extravascular albumin accumulation indicated by Evans Blue dye in the kidney (**A**) and liver (**B**) of rats 24 h after lipopolysaccharide (LPS) or saline administration in combination with different dosages of HAM8101 (n=8 per group). Data are expressed as median and interquartile range. Bold P-values were calculated using Kruskal–Wallis. Pairwise comparisons were made using Mann–Whitney U tests.

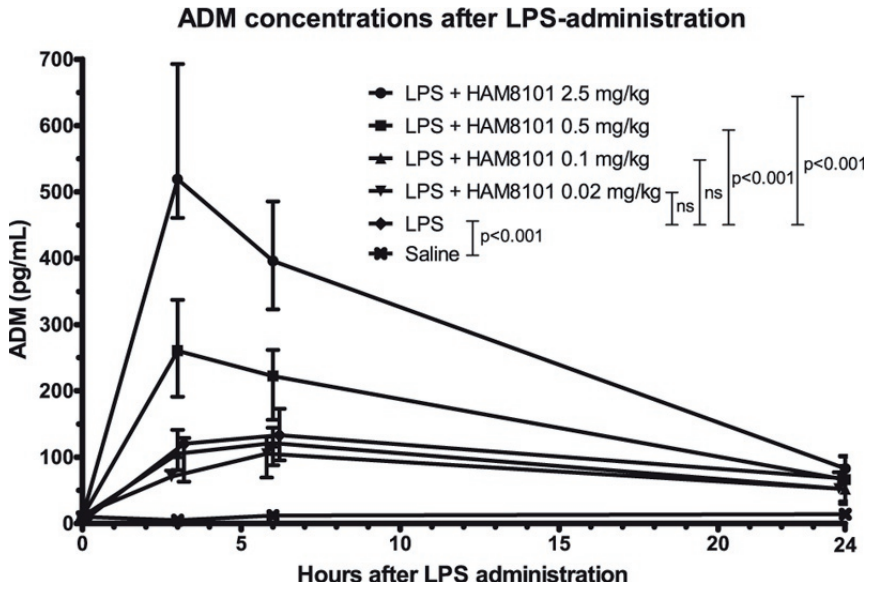

Figure 2. Plasma total ADM concentrations in rats treated with LPS (5 mg/kg) or saline in combination with different dosages of HAM8101 (n=8 per group). Groups were evaluated using two-way ANOVAs (interaction term of group and time) on group pairs using log-transformed data.

HAM8101 improves kidney barrier function during murine sepsis

The effects of HAM8101 on albumin accumulation, VEGF, and angiopoietin-1 expression were investigated in murine CLP-induced sepsis. Representative images of immunohistological stainings are provided in **Figure 3A**. Densitometric evaluation of immunohistological stained slides of renal tissue revealed significantly lower extravascular albumin accumulation with all three dosages of HAM8101 compared with saline-treated CLP animals (78%, 77%, and 77% attenuation for 0.1, 2.0, and 20 mg/kg Adrecizumab, respectively), without HAM8101 dose dependency (**Figure 3B**). Likewise, significantly lower VEGF expression was observed in all HAM8101-treated CLP mice (55%, 45%, 59% attenuation, respectively, **Figure 3C**). Expression of the protective protein angiopoietin-1 was significantly augmented in CLP mice treated with dosages of 0.1 and 2.0 mg/kg HAM8101 (387% and 474% augmentation, respectively, **Figure 3D**), whereas this effect did not reach statistical significance in the 20 mg/kg group (379% augmentation, P=0.065).

Both single- and repeated-dose administration of HAM8101 improve survival during murine sepsis

Finally, the effects of single- and repeated-dose administration of HAM8101 on survival in CLP-induced sepsis were studied in mice, and compared to the effects of the murine analogue HAM1101, which was previously shown to increase survival in the same model[26]. In single-dose administration experiments, CLP caused 90% 7-day mortality, which was significantly improved by both HAM8101 and HAM1101 (50% and 30% mortality, respectively, difference between both antibodies was not statistically significant, **Figure 4A**). In the repeated-dose administration experiments, CLP caused 100% 14-day mortality, which was also significantly improved by the administration of both HAM8101 and HAM1101 (60% and 80% mortality, respectively, no significant difference between both antibodies, **Figure 4B**).

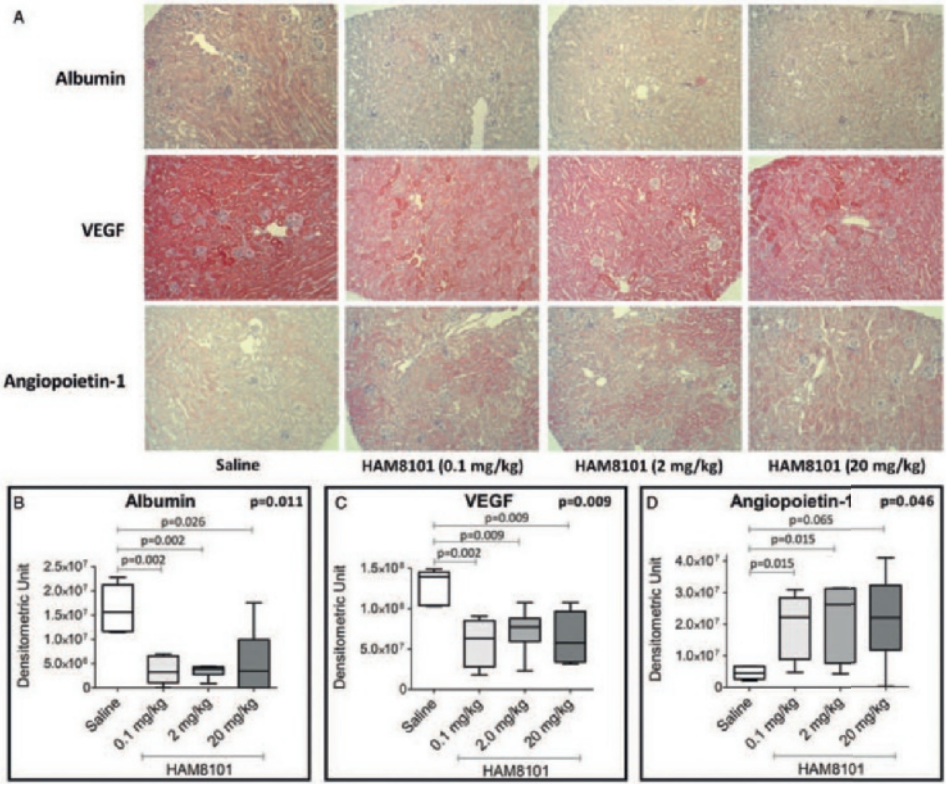

Figure 3. Representative images of immunohistological stainings are provided in the top of the figure (**A**).Extravascular albumin (**B**), vascular endothelial growth factor (VEGF) (**C**), and angiopoietin-1 (**D**) expression in the kidney determined using immunohistochemical analysis 18 h after cecal ligation and puncture, with or without HAM8101 treatment (n=6 per group). Data are expressed as median and interquartile range. Bold P-values were calculated using Kruskal–Wallis tests. Pairwise comparisons were made using Mann–Whitney U tests.

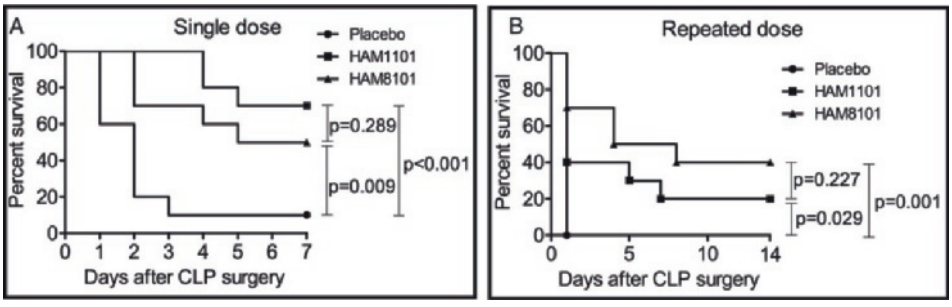

Figure 4. Survival after cecal ligation and puncture (CLP) with our without treatment with single (**A**) and repeated (**B**) doses of placebo, HAM1101, or HAM8101 (n=10 per group). Kaplan–Meier curves are depicted and P-values were calculated using log-rank tests.

Discussion

In the present study, we investigated the effects of the humanized recombinant monoclonal anti-Adrenomedullin antibody HAM8101 (Adrecizumab) on vascular barrier dysfunction and survival in rodent models of systemic inflammation and sepsis. We demonstrate that treatment with HAM8101 prevents LPS-induced vascular hyperpermeability in the kidney. Second, we reveal that HAM8101 improves kidney barrier function during murine sepsis, exemplified by significantly reduced extravascular albumin and VEGF expression, as well as increased expression of the protective protein Ang-1. Finally, the humanized recombinant monoclonal antibody HAM8101 enhanced survival during murine sepsis to a similar extent as the murine anti-ADM antibody HAM1101, which was previously shown to be efficacious[26,27].

Our findings with HAM8101 support previous studies in which beneficial effects of ADM-binding with HAM1101 were demonstrated during murine sepsis, including reduced catecholamine infusion rates, attenuated kidney dysfunction, and improved survival[26,27]. Our finding of reduced renal interstitial edema formation in endotoxemic rats underscores findings from a previous study in which indirect evidence also pointed toward reduced vascular leakage: urine output was shown to be significantly higher in the HAM1101-treated mice, while fluid administration rates were similar[27]. This was accompanied by improved creatinine clearance, urea levels, and less urinary excretion of tubular damage marker neutrophil gelatinase-associated lipocalin (NGAL), which were not measured in the present study[27].

HAM8101 pretreatment attenuated sepsis-induced VEGF expression in the kidney. VEGF is a potent inducer of vascular permeability[30], cell adhesion molecule expression[31], as well as cyto- and chemokine release[32]. Interestingly, increased VEGF concentrations are observed in septic patients, and associated with their fluid balance[33,34], and anti-VEGF treatment improved outcome during models inflammation and sepsis in preclinical studies[35]. HAM8101 enhanced renal expression of angiopoietin-1, a vascular specific growth factor, also involved in the regulation of vascular permeability during sepsis and septic shock[36] that appears to counteract the observed VEGF effects. Angiopoietin-1 levels are also increased in sepsis and septic shock patients, and lower values are associated with increased mortality[37]. Preclinical studies have demonstrated beneficial effects of Angiopoietin-1 on microvascular dysfunction and endothelial barrier function[38,39]. Collectively, our immunohistological

findings of decreased VEGF expression and enhanced angiopoietin-1 expression provide mechanistic support for HAM8101's protective effects on vascular permeability that we observed in rats. As VEGF is also implicated in endothelial dysfunction, it appears plausible that HAM8101 may improve endothelial dysfunction as well, although this remains to be determined.

We show that the survival benefit in murine sepsis is comparable for HAM1101 and HAM8101 for both single and repeated dose administration. This is not unexpected, because HAM8101 is derived from HAM1101, and therefore both antibodies target the exact same epitope. Furthermore, both antibodies were shown to be ideally cross-reactive among all mammalian species tested (mice, rats, dogs, pigs, and humans; unpublished data). Also, as mentioned previously, both antibodies, despite their high affinity for ADM, only partially and to a similar extent inhibit the ADM-induced cAMP response in CHO cells overexpressing the ADM (CRLR/RAMP3) receptor[26].

Of particular interest is the fact that HAM8101 administration led to a dose-dependent increase in total ADM levels in our study. This is in line with the results from our recently performed phase I study in healthy volunteers[40], and we have formulated a hypothesis on this aspect of HAM8101 therapy[41]. Briefly, we believe that it involves elongation of ADMs half-life by binding with the non-neutralizing antibody HAM8101, providing protection from N-terminal proteolytic degradation. Second, we hypothesize that ADM distribution is shifted from another compartment (likely the interstitium) toward the circulation, based upon the fact that ADM can normally diffuse freely over the endothelial barrier (6kD peptide), whereas it likely cannot after binding to HAM8101. This is supported by data from our phase I study, showing a low volume of distribution (+/- 100mL/kg)[40]. Nevertheless, more research is required before definite conclusions can be drawn on the purported mechanism of action of HAM8101.

It could be argued that the HAM8101-induced increase in circulating ADM levels negatively influences blood pressure, as high dosages of ADM have been reported to exert vasodilatory effects[21–25]. We cannot draw direct conclusions on this possible untoward effect, because we did not measure blood pressure in our experiments. It has to be kept in mind, though, that the increase of circulating ADM does not represent free ADM, but ADM complexed with Adrecizumab. As alluded to in the section above, this complex is too large to

cross the endothelial barrier, and therefore cannot exert vasodilation through direct effects on vascular smooth muscle cells. Furthermore, data from previous studies do not support detrimental effects on blood pressure either. For instance, pretreatment with the murine form of the antibody (HAM1101) resulted in reduced vasopressor infusion requirements, which argues against hypotensive effects of the antibody, at least under inflammatory conditions[27]. Finally, no effects on blood pressure or heart rate were observed in our phase I study[40].

A strength of the current work is the use of different animal species, sepsis(-like) models, and end-points. Several limitations also need to be addressed. Although our results are encouraging, it needs to be acknowledged that HAM8101 and HAM1101 were administered 5 min prior to LPS-administration/CLP-surgery in our experiments, to demonstrate proof of principle. As time dependency of the effects was not investigated, it remains to be determined whether HAM8101 also exerts beneficial effects when treatment is delayed, which is more representative of the clinical setting in sepsis patients. Moreover, measurements of vascular permeability were only performed at one time-point in each study and were restricted to the kidney and liver. It would also be of interest to evaluate whether HAM8101 exerts similar effects in the lung, as vascular leak in this organ is the major cause of acute respiratory distress syndrome in sepsis patients. Another limitation is the fact that the CLP-induced kidney barrier dysfunction experiment did not include a sham control group.

Conclusion

Pretreatment with the humanized anti-ADM antibody HAM8101 improves vascular barrier function and survival in rodent models of systemic inflammation and sepsis. The promising findings from the current animal experiments warrant further research in humans. Currently, a phase I study (ClinicalTrials.gov identifier NCT02991508) is in its final stages and a phase II study in septic patients is planned.

Ethics approval and consent to participate

Study protocol approval was obtained from the appropriate governing institutes (approval numbers: Preclinics: 2347-35-2015 issued by Landesamt für Umwelt, Gesundheit und Verbraucherschutz Brandenburg; Phenos: 33.9-42502-04-12/0846 issued by Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit).

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Chapter 10

The non-neutralizing anti-adrenomedullin antibody Adrecizumab improves hemodynamics and attenuates myocardial oxidative stress in septic rats

Alice Blet, Benjamin Deniau, Christopher Geven, Malha Sadoune, Anaïs Caillard, Paul-Robert Kounde, Evelyne Polidano, Peter Pickkers, Jane-Lise Samuel and Alexandre Mebazaa

Submitted

Abstract

Background

Sepsis represents a major health issue, with persisting high morbidity and mortality rates. Cardiovascular dysfunction occurs frequently during sepsis. Adrenomedullin (ADM) has been identified as a key mediator in vascular tone regulation. A non-neutralizing anti-ADM antibody, Adrecizumab, may improve hemodynamic dysfunction during murine, cecal ligation and puncture (CLP)-induced septic shock. Our objective was to determine the role of Adrecizumab on hemodynamics in a rat model of sepsis.

Methods

CLP-surgery was performed in Wistar male rats to induce sepsis. Single blinded dose of Adrecizumab (2 mg/kg) or placebo was injected IV 24 hours after the surgery and norepinephrine (NE) was infused as standard of care. There were > 7 animals per group. Invasive blood pressure (BP) and cardiac function (by echocardiography) were assessed until 3 hours after Adrecizumab injection.

Results

A single therapeutic injection of Adrecizumab in septic rats induced rapid beneficial hemodynamic effects with an increase in systolic BP in CLP-Adrecizumab rats versus untreated-CLP rats ($p=0.049$). The shortening fraction did not differ in both untreated-CLP and CLP-Adrecizumab groups. On the other hand, cardiac output increased during the 3 hours after a single dose of Adrecizumab compared to untreated-CLP rats ($p=0.006$). Adrecizumab administration resulted in hemodynamic effects similar to continuous administration of norepinephrine. Three hours after Adrecizumab administration, there was no change in the inflammatory phenotype (TNFα, IL-10) in the myocardium of the septic rats. In contrast, myocardial free radical production (DHE) was decreased in CLP-Adrecizumab vs. untreated CLP rats ($p<0.05$).

Conclusions

In a rat model of sepsis, a single therapeutic injection of Adrecizumab rapidly restored hemodynamic parameters and attenuated myocardial oxidative stress. Currently, a proof-of-concept and dose-finding phase II trial (Adrenoss-2) is ongoing in patients with septic shock and elevated concentrations of circulating bio-ADM.

Introduction

Despite advances in resuscitation and infectious diseases management, sepsis remains one of the leading causes of death worldwide[1,2]. Sepsis is characterized by disturbed vascular integrity, the presence of life-threatening organ dysfunction due to a dysregulated response of the body to infection[2]. Today, vasopressor therapy is one of the cornerstone of sepsis treatment, however, vasopressor use do not restore vascular integrity and could even lead to harmful effects and impair prognosis[3].

Adrenomedullin (ADM), a 52 amino acids peptide hormone[4,5], has been proposed as a pivotal mediator of vascular dysfunction in sepsis[6,7]. On the one hand, ADM can act as a vasodilator, decrease peripheral vascular resistance, and increase cardiac output[8]. On the other hand, ADM has beneficial effects, as it reduces capillary hyperpermeability in preclinical studies with models of septic shock[9,10]. Recently, a model has been proposed, which explains these different activities of ADM as a function of its compartmental localization[11]: in the interstitium, ADM acts on vascular smooth muscle cells to induce vascular relaxation, whereas in the blood circulation ADM promotes the stabilization of the endothelial barrier. The ADM pathway acts through ADM receptors made of one CRLR subunit and one RAMP 2 or 3 subunit.

In patients with sepsis and septic shock, elevated plasma concentration of biologically active ADM (bio-ADM) is associated with disease severity, organ dysfunction, and it is a strong prognosticator for 28-day mortality[12–15]. Of interest and related to its vascular effects, high plasma concentrations of bio-ADM is correlated to vasopressor use[12–15]. Therefore, modulation of ADM activity could be of therapeutic potential during sepsis to restore hemodynamics and improve clinical outcome[16]. Adrecizumab (HAM8101) is a humanized non-neutralizing monoclonal antibody directed against the N-terminus of ADM that only partially inhibits ADM activity. Intravenous Adrecizumab administration leads to an immediate and substantial increase of plasma ADM concentration, thereby enhancing the endothelium-stabilizing effect of ADM[17]. In a mouse model of sepsis (cecal ligation and puncture, CLP), pretreatment with Adrecizumab increased survival while other antibodies directed against different epitopes of ADM (causing greater or complete inhibition of ADM signaling) did not[17,18]. In addition, pretreatment with Adrecizumab led to numerous improvements including reduced catecholamine and fluid requirements, as well as improvement

of renal function[19]. Given the role of ADM in vasodilation and capillary leakage, the objective of this work was to explore the hemodynamic response to Adrecizumab therapeutic treatment, by partial ADM inhibition, in a rat model of sepsis. We hypothesized that by this treatment a rapid and sustained beneficial response could be achieved in septic shock.

Material and methods

Animals and sepsis model

Two months old male Wistar rats weighing 350 to 450 g were obtained from Janvier (St Berthevin, France). All experiments were conducted in accordance with the National and European Institutes of Health guidelines for the use of laboratory animals and were approved by the local animal research ethics committee (Lariboisière-Villemin, Paris, France; 77-2014 -ceea9).

All animals were anesthetized using ketamine hydrochloride (90 mg/kg) and xylazine (9 mg/kg) intraperitoneally. For induction of polymicrobial sepsis, CLP was performed as previously described[20]. A ventral midline incision (1 cm) was made to allow exteriorization of the cecum. The cecum was then ligated just below the ileocecal valve and punctured once with an 18-gauge needle. The abdominal cavity was closed in two layers and rats were given fluid resuscitation (3 ml/100g of body weight of saline injected subcutaneously). A sham operation was performed by isolating the cecum without ligation or puncture. Twenty-four hours later, rats were split into several groups. CLP animals were randomized in 5 subgroups to a single blinded i.v. dose of Adrecizumab (2 mg/kg in 1.5 mL) or placebo (1.5 mL of PBS) through the jugular vein and norepinephrine (NE), for either 30 minutes or continuously as the standard of care for hemodynamic management, or not: CLP, CLP-Adrecizumab, CLP-cNE (with continuous NE), CLP-cNE-Adrecizumab (Adrecizumab + continuous NE) and CLP-NE (with NE infusion during 30 min). Norepinephrine was administrated at the dose of 1 µg/kg/min. There were at least 7 rats per group (except for the CLP group with norepinephrine infusion during only 30 min, n=4). Among CLP rats, 20 died before administration treatment of either Adrecizumab or placebo and hemodynamic exploration. Sham animals received neither Adrecizumab nor NE. The experimentation protocol is summarized in **Figure 1**.

Briefly, 24 hours after CLP procedure, rats were anesthetized with ketamine hydrochloride (90 mg/kg) and xylazine (9 mg/kg)[20], and placed in supine

position. Animals were intubated with a catheter (16G) and ventilated using a rodent ventilator with respiratory rate = 53.5 * 0.26 weight, tidal volume = 6.2 * 1.01 weight. Rectal temperature was maintained throughout the protocol at 37–37.5 °C by a heating mat. Catheters were inserted into left jugular vein to administer antibody or placebo and into the right carotid artery to monitor blood pressure.

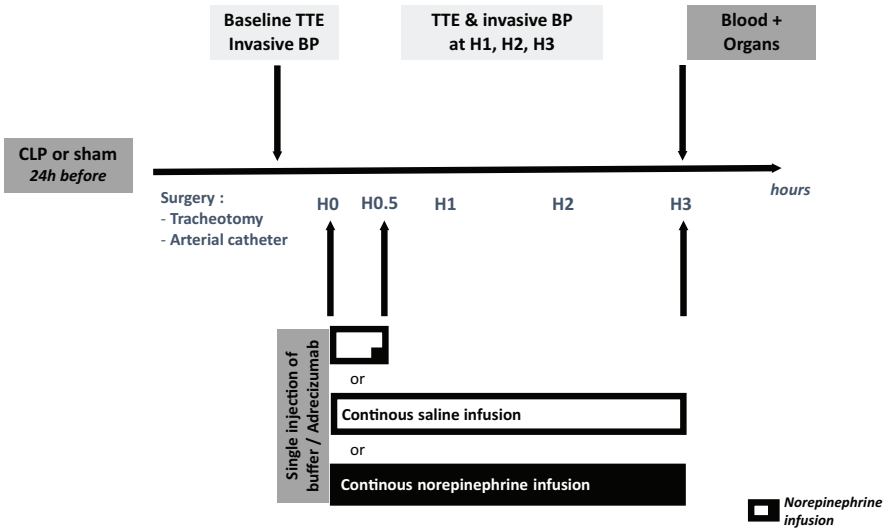

Figure 1. Experimental protocol

Hemodynamics and cardiac function monitoring

Cardiac function was assessed by transthoracic echocardiographic examination at baseline and every hour during the next 3 hours of the experiment using GE Healthcare Vivid 7 Ultra-sound System equipped with a high frequency (14-MHz) linear probe. All examinations were recorded digitally and stored for subsequent off-line analysis as described by Milliez et al.[21]. Invasive blood pressure (BP) measurements were performed after catheter insertion and every hour during the next 3 hours.

Assessment of organ inflammatory response and oxidative stress

EDTA blood was collected from the left carotid artery at the end of the experiment, centrifuged at 3500 rpm for 15 min at 4°C, and plasma was stored at -80°C until measurement of several analytes. Bio-ADM was measured as described previously[17]. At the end of the protocol, rats were sacrificed and organs (heart, lung, liver and left kidney) weighed. The heart was transversely divided into 2 parts. The base was embedded into Tissue-Tek optimal cutting

temperature (OCT) compound (Sakura Finetek, France) and frozen in liquid nitrogen, and stored at -80°C until use for DHE staining; the other part of the heart was snap-frozen in liquid nitrogen for RT-PCR and Western Blot analysis. Other organs specimens (lung, liver, kidney, brain, aorta and muscle) were collected and snap frozen in liquid nitrogen. All samples were stored at -80°C until further analysis.

Gene expression analysis

Total RNA was isolated from tissues using the RNeasy Mini Kit® (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions and reverse transcribed using QuantiTect® Reverse Transcription (Qiagen, Courtaboeuf, France). Then, real-time polymerase chain reaction was performed with LightCycler96 (Roche Diagnostics, Meylan, France) using the FastStart Essential DNA Green Master® (Roche Diagnostics, Meylan, France). mRNA levels for genes of interest were normalized to that of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expressed as the relative change compared with the control samples. The sequences of the primers used are reported in **Suppl. Table 1**.

Protein analysis

For western blot analysis, tissues were homogenized in cell lysis buffer (50 mM Tris HCl at pH 7.4, 1 mM EDTA, and 150 mM NaCl). After centrifugation, soluble proteins were quantified using the Pierce BCA Protein Assay Kit (Thermo Fischer Scientific, Courtaboeuf, France). Proteins (30 µg) were separated on 10-12% SDS-PAGE gels and were transferred onto nitrocellulose membranes (Protan Paris, France). Blots were probed overnight at 4°C with the following primary antibodies directed against the following: phosphorylated and total Akt (Ser473) (1:1000; #9271 and #9272; Cell Signaling, Ozyme, France), p62 (1:1000; ab56416; Abcam, United Kingdom), HIF1α (1:1000; PAI 16601; Thermo Scientific, Massachusetts, USA), and GAPDH (1:5000; Millipore, Molsheim, France). Blots were incubated with goat anti-rabbit (1:5000; Sigma-Aldrich) or sheep anti-mouse peroxidaseconjugated antibodies (1:10,000; GE Healthcare) for 1 h at room temperature. Chemiluminescent signals (ECL Plus; GE Healthcare) were recorded using an LAS 3000 system (Fuji, Courbevoie, France) and were quantified using MultiGauge V2.02 software (Fuji). The results are expressed as arbitrary units (AU) obtained from the ratio between the densitometric units of the protein under study and the GAPDH densitometric value.

Histological and histochemical analyses

Seven µm cross sections were stained with hemalun and eosin and examined by bright-field microscopy at 20X magnification. Cardiac cryostat cross-sections (7 µm) of the ventricles were incubated with Dihydroethidium (DHE; Sigma-Aldrich) (37 µM) for 30 min in a dark humidified chamber[22]. Acquisition of fluorescent images of ethidium bromide with Leica fluorescence microscope was performed under identical setting whatever the block tissue. The stained area was measured with IPLab software and expressed as a percentage of area of interest (% of ROI).

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, California). Comparison between groups was performed by 2-way ANOVA or Kruskal Wallis followed by Dunn's Multiple Comparison Test as appropriate. Mann-Whitney U test was used to compare baseline hemodynamics parameters. A p-value < 0.05 was considered statistically significant.

Results

At the time of hemodynamic resuscitation, clinical signs of sepsis (reduced motor activity, lethargy, shivering, piloerection and hunched posture) were only present in CLP rats as expected. Furthermore, post-mortem examination of the abdominal cavity of all CLP rats showed varying degrees of peritonitis with a gray-black dilated cecum and purulent and malodorous peritoneal fluid. There was no difference in heart, lung, liver and left kidney weights between sham and CLP rats (**Suppl. Table 2**).

Effects of Adrecizumab on hemodynamics

Before initiation of hemodynamic resuscitation, all CLP rats presented with altered hemodynamics including a markedly lower mean BP (95 ± 3 versus 112 ± 3 mmHg, p = 0.0006) and a slight increase in cardiac output (160 ± 5 versus 133 ± 8 µL/min, p = 0.018) compared to sham rats (**Suppl. Figure 1**). In untreated CLP rats, hemodynamics remained altered during the 3 hours of experimentation (**Figure 2**). A single dose of Adrecizumab (CLP-Adrecizumab rats) without norepinephrine rapidly increased systolic blood pressure (p = 0.049 vs. untreated CLP). Adrecizumab injection also tended to improve diastolic and mean blood pressures and LV shortening fraction, although these did not reach statistical significance (**Figure 2**).

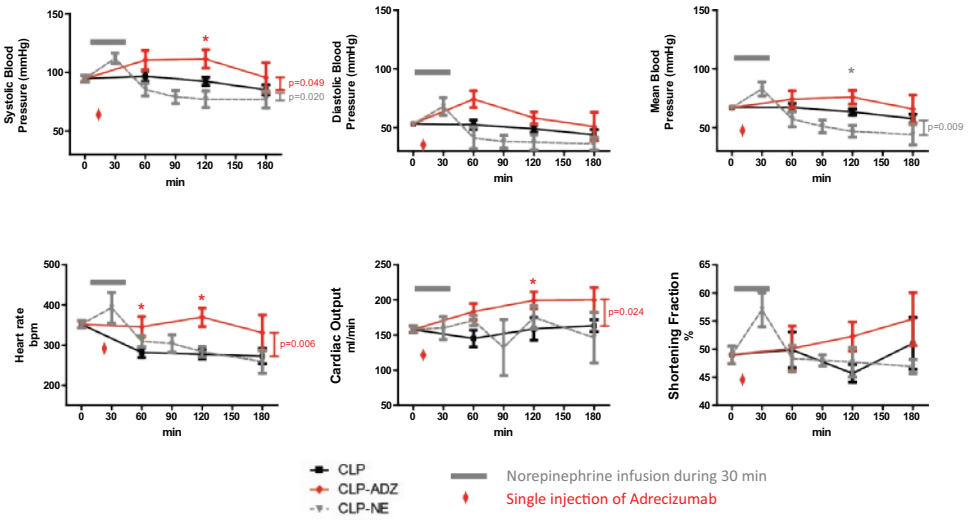

Figure 2. Hemodynamic parameters measured 24 hours after sepsis induction by CLP and then 60, 120 and 180 min after the injection of Adrecizumab or placebo. CLP rats are represented by the black line, CLP-Adrecizumab rats by the red line and CLP-NE rats by the grey line. Two-way ANOVA was used to compare between-group differences. * p<0.05.

In addition, cardiac output and heart rate significantly increased during the 3 hours after single dose administration of Adrecizumab compared to untreated CLP rats (p=0.006 and p=0.024 respectively; **Figure 2**). During the protocol, restoration of systolic blood pressure and improvement in cardiac output were similar in CLP rats receiving continuous NE infusion and those receiving a single dose of Adrecizumab (**Suppl. Figure 2**). Furthermore, the addition of a single injection of Adrecizumab to continuous NE infusion had a similar effect as NE on hemodynamic parameters and did not lead to unwanted further vasoconstriction (**Suppl. Figure 2**). Of note, short-term (30 min) administration of NE only transiently improved hemodynamics and parameters either returned to baseline values (e.g. cardiac output and heart rate) or worsened (e.g. blood pressure) compared to baseline, after stopping NE infusion (**Figure 2**).

Adrecizumab and metabolic changes in sepsis

Concerning circulating bio-ADM, levels were low in sham animals (14.6 ± 2.1 pg/mL), while a strong elevation was observed in untreated CLP rats (289.7 ± 42.2 pg/mL, p<0.05 vs. sham; **Figure 3**). Administration of Adrecizumab further increased plasma bio-ADM concentration (1193 ± 349.8 pg/mL, p<0.05 vs. sham; **Figure 3**).

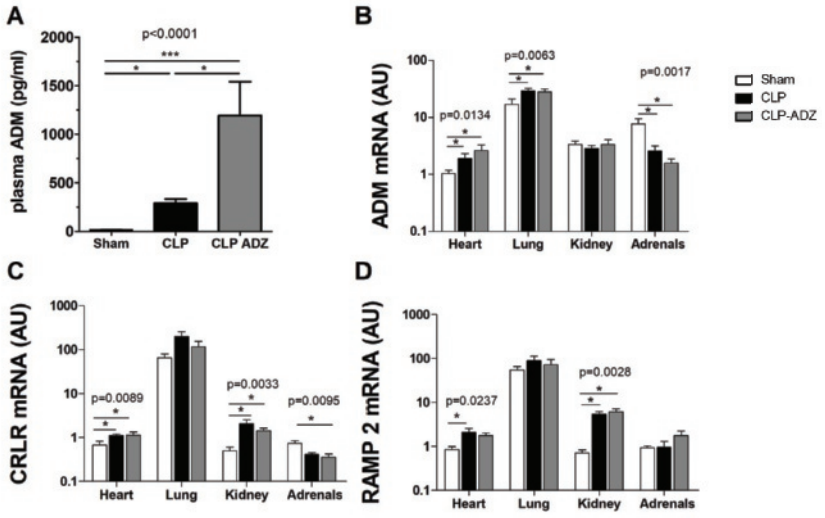

Figure 3. Adrenomedullin plasma level (pg/ml) (**A**). Expression of adrenomedullin (ADM) mRNA (**B**) and its receptor (CRLR, RAMP2) (**B, C**) in the heart, lung, kidney and adrenals. These measurements were performed 3 hours after the Adrecizumab injection and 24 hours after the induction of sepsis. Groups were compared using the Kruskal-Wallis test followed by Dunn's multiple comparison test.

Myocardial and lung expression of ADM were increased in untreated CLP versus sham (heart: 2.17 ± 0.4 vs. 0.9 ± 0.1, p<0.05; lung: 29.1 ± 3.1 vs. 13.4 ± 2.2, p<0.05; **Figure 3**) and remained high 3 hours after single Adrecizumab injection (heart: 2.64 ± 0.6, p<0.05 vs. sham; lung: 28.1 ± 3.6, p<0.05 vs. sham). In contrast, adrenal expression of ADM decreased in both untreated CLP and in CLP-Adrecizumab rats versus sham (respectively: 2.57 ± 0.6, 1.58 ± 0.3 vs. 7.67 ± 1.9, p<0.05). No change of ADM expression was observed in lung.

Expression of the ADM receptor components CRLR and RAMP2 was by far highest in the lung, 50-100 fold higher than in heart, kidneys and adrenals. Myocardial and kidney expression of CRLR were increased in untreated CLP versus sham (heart: 1.11 ± 0.1 vs. 0.54 ± 0.1, p<0.05; kidney: 2.08 ± 0.5 vs. 0.51 ± 0.1, p<0.05; **Figure 3**) and remained high 3 hours after single Adrecizumab injection (heart: 1.14 ± 0.2, p <0.05 vs. sham; kidney: 1.42 ± 0.2, p <0.05 vs. sham). In contrast, adrenal expression of CRLR decreased significantly in CLP-Adrecizumab versus sham (0.36 ± 0.06 vs. 0.74 ± 0.11, p <0.05) and non-significantly in untreated CLP (0.42 ± 04). No change of CRLR expression was observed in lung.

Myocardial and kidney expression of RAMP 2 were increased in untreated CLP versus sham (heart: 2.12 ± 0.5 vs. 0.89 ± 0.2, $p<0.05$; kidney: 5.42 ± 0.8 vs. 0.71 ± 0.1, $p<0.05$; **Figure 3**) and remained high 3 hours after single Adrecizumab injection (heart: 1.77 ± 0.3, ns vs. sham; kidney: 6.14 ± 1.1, $p<0.05$ vs. sham). No change of RAMP 2 expression was observed in lung and adrenals.

Regarding myocardial inflammatory markers, the expression of the pro-inflammatory marker TNFα, the anti-inflammatory marker IL-10 and CD68, a marker of macrophage activation, were all increased in CLP rats. Myocardial levels of those markers were not altered in CLP-Adrecizumab rats. Moreover, myocardial BNP mRNA was upregulated in untreated CLP rats and remained high in CLP-Adrecizumab. In addition, p62, an inflammatory marker, was increased in the heart of untreated CLP and CLP-Adrecizumab compared with sham rats (**Figure 4**). **Suppl. Figure 3** also shows that myocardial BNP mRNA was up regulated in CLP and remained high in CLP-Adrecizumab.

Figure 4 also shows that the Akt phosphorylation level, a myocardial survival pathway, was markedly increased in the myocardium of CLP-Adrecizumab rats 24 hours after the onset of sepsis ($p<0.05$). Concerning myocardial oxidative stress, the CLP-induced 10-fold elevation of DHE that was blunted in CLP-Adrecizumab rats ($p<0.05$) (**Figure 5**).

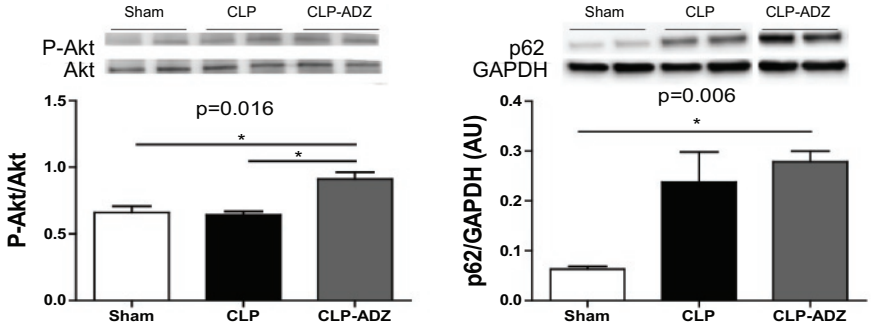

Figure 4. Western Blot of cardiac P-Akt/Akt and p62. Groups were compared using the Kruskal-Wallis test followed by Dunn's multiple comparison test.

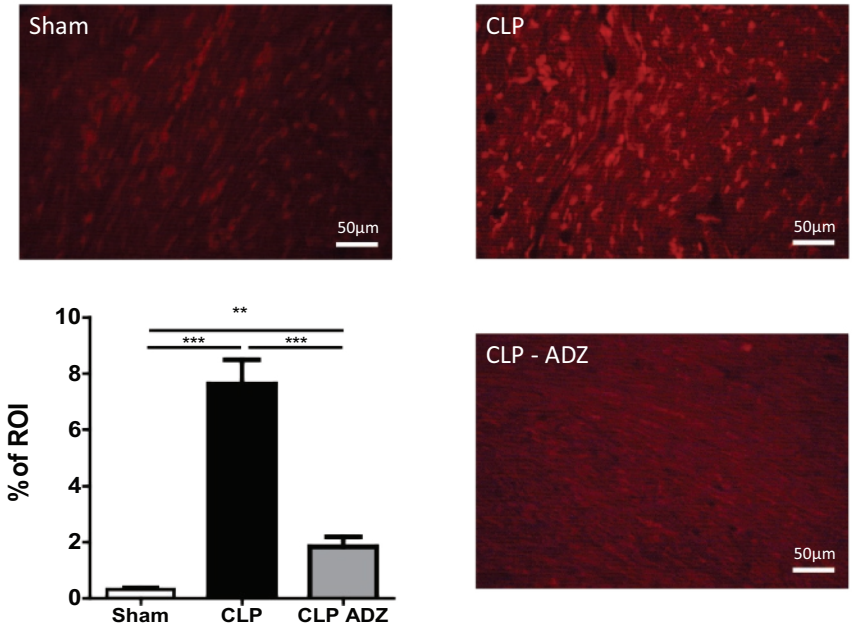

Figure 5. Decreased ROS production in the septic myocardium 3 hours after Adrecizumab administration. Dihydroethidium (DHE; Sigma-Aldrich) staining was used to evaluate the in situ levels of superoxide anion in the myocardium. Data are expressed as a percentage of region of interest (% of ROI). Groups were compared using the Kruskal-Wallis test followed by Dunn's multiple comparison test.

Discussion

The present study shows that a single therapeutic injection of Adrecizumab in septic rats induced rapid hemodynamic benefits and a marked reduction in tissue oxidative stress. Indeed antibodies directed against ADM, an endogenous vasodilator peptide had similar hemodynamic effects as continuous administration of norepinephrine.

Our study demonstrates that in a model of sepsis, a single injection of Adrecizumab rapidly restored blood pressure and cardiac output. ADM has been proposed to be one of the pivotal mediators of vascular dysfunction in sepsis. In patients with sepsis or septic shock, high plasma bio-ADM levels are related to a worse prognosis and higher need for vasoconstrictors[12]. As recently described, circulating bio-ADM easily diffuses from the lumen of the vessels to the interstitium to act on vascular smooth muscle cells and reduce vascular tone[11]. Adrecizumab was described to improve blood pressure when given as preventive therapy before induction of sepsis[19]. Herein we showed that, a single injection of Adrecizumab restored blood pressure, 24 hours after induction of peritonitis and septic shock in rats. The early benefit of a single injection of Adrecizumab on blood pressure (**Figure 2**) was likely related to the rapid binding of Adrecizumab to plasma bio-ADM, hence preventing its diffusion to the interstitium.

Our study further shows that a single injection of Adrecizumab was associated with a sustained improvement in cardiac output in septic rats. This is the first demonstration that Adrecizumab, likely acting as a scavenger of circulating bio-ADM, not only improves blood pressure but also improves systemic perfusion. Improvement in cardiac output might be related, at least partially, to the higher heart rate after single administration of Adrecizumab. The data also indicated that Adrecizumab improved left ventricular shortening fraction, though not statistically significant, in septic rats. In septic shock patients, hemodynamics, and especially blood pressure is usually restored by continuous administration of vasopressors such as catecholamine, vasopressin or angiotensin[23]. The restoration of blood pressure and improved cardiac output was similar to continuous administration of norepinephrine. This novel approach might be safer as it avoids the long-lasting administration of vasopressors, possibly associated with deleterious effects on outcome[3]. Therefore, norepinephrine substitution by Adrecizumab might be of interest. Our data show that beneficial effects of Adrecizumab on blood pressure at the

dose of 2 mg/kg might decrease after 2 hours though benefits on cardiac output appeared to be maintained. Further studies should test greater doses. Hence, the present preclinical work confirms and extends the short-term safety and efficacy profile of the non-neutralizing ADM-binding antibody Adrecizumab in line with improved renal function and survival previously described[17,18]. This led to pursue the program in human septic patients. Adrecizumab is still investigational. A phase 2 trial, AdrenOSS-2, has started in December 2017 to assess safety and efficacy of single injection of Adrecizumab (2 or 4 mg/kg) in patients with septic shock (NCT03085758). AdrenOSS-2 trial is one of the first personalized medicine trials in septic shock patients.

Regarding CRLR, RAMP 2 and ADM expression in various tissues. Our data confirmed that ADM pathway is highly present in lungs compared to other organs including heart, kidney and adrenal glands[24,25]. Twenty-four hours of peritonitis and septic shock induced changes in ADM pathway with a greater expression in ADM in the heart and lung, in CRLR and RAMP 2 in the heart and kidney. Whereas, in adrenals, where ADM was first described[4], sepsis decreased ADM and CRLR expressions. The single injection Adrecizumab had no effect on ADM pathway. Peritonitis and septic shock upregulated inflammation and survival pathways in the heart and in other organs. Those changes were unaffected as early as 3 hours after a single Adrecizumab injection. The observation-time was likely too short to see any significant changes on ADM pathway and tissue inflammation and longer observation period being needed. By contrast, single Adrecizumab injection succeeded to rapidly and markedly reduce myocardial oxidative stress. The mechanisms through which Adrecizumab reduced myocardial oxidative stress are not fully understood, although anti-apoptotic, anti-inflammatory and anti-oxidative properties previously described might be involved[11,17]. The improvement of myocardial function may also be related to the Adrecizumab-induced reduction in myocardial oxidative stress[26,27].

Conclusion

Therapeutic treatment with the ADM-binding antibody Adrecizumab improves short-term hemodynamic parameters and attenuates myocardial oxidative stress in rat polymicrobial sepsis. Currently, a proof-of-concept and dose-finding phase II trial is ongoing in patients with septic shock and elevated concentration of circulating bio-ADM.

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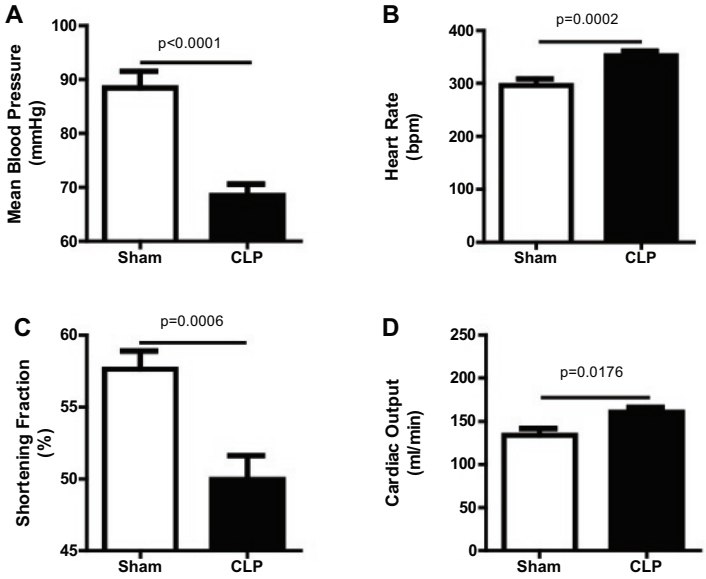
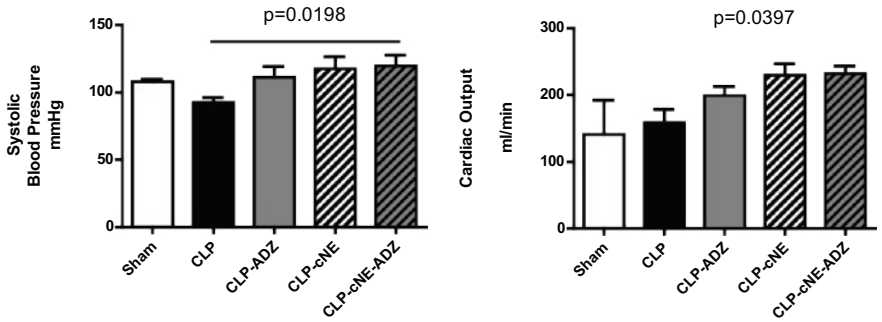
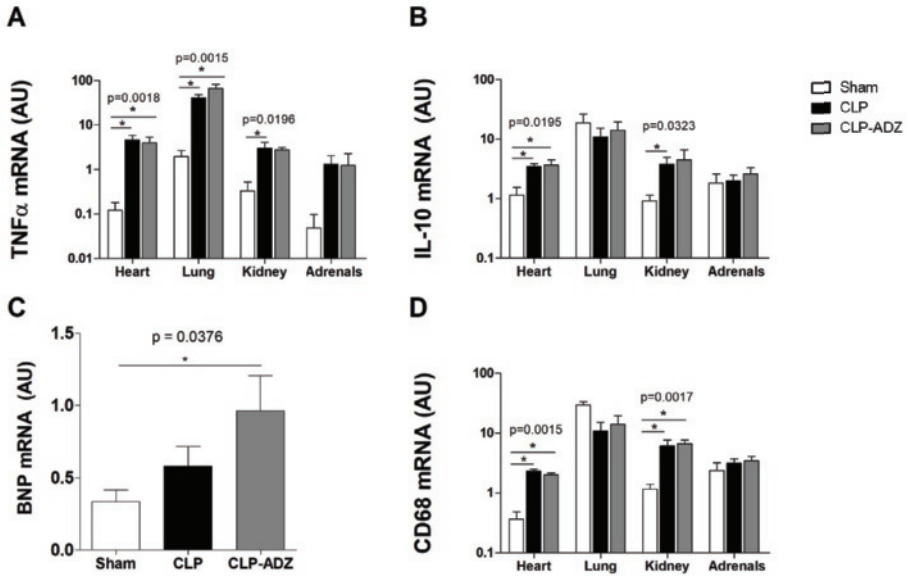


Suppl. Figure 1. Hemodynamics 24 hours after CLP and prior to antibody injection. Average arterial pressure (mmHg) (**A**). Heart rate (bpm) (**B**). Fraction of shortening (**C**). Cardiac flow (ml / min) (**D**). Groups were compared using the Mann-Whitney U test were used.



Suppl. Figure 2. Hemodynamic parameters measured 24 hours after sepsis induction by CLP and 120 min after single injection of Adrecizumab or placebo. cNE means continuous administration of norepinephrine. Groups were compared using the Kruskal-Wallis test followed by Dunn's multiple comparison test.



Suppl. Figure 3. Expression of cytokine mRNA in various organs (**A, B**). Myocardial expression of BNP (**C**). Expression of CD68 mRNA in various organs (**C**). These measurements were performed 3 hours after the Adrecizumab injection and 24 hours after the induction of sepsis. Groups were compared using the Kruskal-Wallis test followed by Dunn's multiple comparison test.

Suppl. Table 1. Primer sequences

	Gene	Access GenBank	Forward sequence	Reverse sequence
ADM	Adm	NM_012715.1	TTCTCATCGCAGTCAGTCTTGG	CGCTTGTAGTTCCCTCTTCCC
BNP	Nppb	NM_031545.1	TAGCCAGTCTCCAGAACAATCCA	AAACAACCTCAGCCCGTCAC
CD68	Cd68	NM_001031638.1	GCCCTCACCAAGTCCTAGTC	GATGTCGGTCCTGTTTGAATCCA
CRLR	Calcrl	NM_012717.1	TCAGCTCAGACACTCATCTCCTC	CACCTCCTCAGCAACCTTTCC
GAPDH	Gapdh	NM_017008.4	GTTCAACGGCACAGTCAAGG	ACTCCACGACATACTCAGCAC
IL10	IL10	NM_012854.2	CCTGCTCTTACTGGCTGGAG	TGTCCAGCTGGTCCTTCTTT
RAMP2	Ramp2	NM_031646.1	TTGTGGTGTGGAGGAGTAAAGAC	GTGGGAAGGATGGGAGTAAGTG
TNF	Tnf	NM_012675.3	ACTCCCAGAAAAGCAAGCAA	CGAGCAGGAATGAGAAGAGG

Suppl. Table 2. Organ weight/body weight

	Sham	CLP	CLP -ADZ	CLP -cNE	CLP -cNE -ADZ	CLP -NE	p-value
Heart/body weight * 1000	3.33 ± 0.09	3.17 ± 0.08	3.13 ± 0.05	3.28 ± 0.06	3.26 ± 0.06	3.07 ± 0.05	ns
Lung/body weight * 1000	4.02 ± 0.15	3.92 ± 0.08	4.13 ± 0.11	3.86 ± 0.11	3.79 ± 0.20	4.83 ± 0.79	ns
Kidney/body weight * 1000	3.51 ± 0.14	3.97 ± 0.13	3.87 ± 0.18	3.74 ± 0.18	3.80 ± 0.19	3.51 ± 0.25	ns
Liver/body weight * 1000	37.53 ± 1.33	36.80 ± 1.30	35.10 ± 0.95	35.16 ± 1.36	32.93 ± 1.01	33.16 ± 0.64	ns

Data are presented as mean ± Standard Error of Mean (SEM). Comparison between groups was performed by Kruskal Wallis analysis followed by Dunns test. A p-value of < 0.05 was considered statistically significant. *Groups: CLP, cecal legiation and puncture; CLP-ADZ, CLP-Adrecizumab; CLP-cNE, CLP-continuous norepinephrine infusion; CLP-cNE-ADZ, CLP-continuous norepinephrine infusion-Adrecizumab; CLP-NE, CLP-norepinephrine during 30 min.*

Chapter 11

Preclinical safety evaluation of the adrenomedullin-binding antibody Adrecizumab in rodents, dogs and non-human primates

Christopher Geven, Matthijs Kox, Alice Blet, Alexandre Mebazaa, Mathias Schroedter, Joachim Struck, Andreas Bergmann and Peter Pickkers

Submitted

Abstract

Adrenomedullin (ADM) is a vasoactive peptide in sepsis. The non-neutralizing ADM-binding antibody Adrecizumab improved outcome in animal models of systemic inflammation and sepsis. Herein, we evaluated the preclinical safety of Adrecizumab in various animal species. First, Wistar rats received vehicle, 100, 200 or 400 mg/kg/day of Adrecizumab intravenously (n=20 each) on days 1, 4, 8 and 14. An additional set of rats received vehicle or 400 mg/kg/day (n=10 each) on the same days and were followed for 42 days. For toxicokinetics, satellite animals received vehicle (n=6), 100, 200, or 400 mg/kg/day Adrecizumab intravenously (n=18 each). A hemodynamic study was performed in Beagle dogs (n=3) receiving vehicle (day 1), 2 mg/kg (day 3), 10 mg/kg (day 5), 50 mg/kg (day 8) and 10 mg/kg Adrecizumab intravenously (day 29). In final experiments, cynomolgus monkeys received vehicle, 25, 50 or 100 mg/kg/day Adrecizumab intravenously (n=6 each) on days 1, 4, 8 and 14. Additional groups of monkeys received vehicle or 100 mg/kg/day Adrecizumab intravenously (n=4 each) on the same days and were followed for 42 days. No mortality or moribund conditions occurred and no toxicologically relevant effects were attributed to Adrecizumab. Adrecizumab significantly increased circulating concentrations of its target peptide ADM, consistent with previous studies and mechanistically relevant. Toxicokinetic analyses showed immediate and dose-dependent peak concentrations, slow elimination and no gender differences. In conclusion, intravenous, repeated administration of high doses of Adrecizumab appeared well-tolerated across species. These results pave the way for further investigation of Adrecizumab in humans (intended dose of 2 mg/kg).

Introduction

Sepsis is a major worldwide health issue, with global estimates of approx. 30 million episodes and over 5 million deaths annually[1]. It is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection[2]. Endothelial dysfunction is a major pathogenic hallmark of sepsis, and tissue edema resulting from breakdown of vascular endothelial barriers is generally regarded as a key mechanism in widespread lethal organ dysfunction[3-5].

Adrenomedullin (ADM) is a vasoactive peptide relevant in the context of sepsis[6]. ADM levels are increased in sepsis patients and associated with organ dysfunction, vasopressor requirements and mortality[7,8]. These observations may imply that ADM plays a detrimental role in sepsis. However, no causal relationships can be assumed from these observational studies, and increased levels of ADM could also indicate a failing protective response. Mechanistic in vitro and in vivo studies have shown that ADM exerts strong stabilizing effects on the vascular endothelium through binding with ADM-receptors present on endothelial cells that line the inside of blood vessels[9-12], thereby preventing capillary leakage. In addition, anti-inflammatory and antibacterial effects have also been attributed to ADM 6. Other studies have shown that ADM is a potent vasodilator and can cause hypotension (in vivo, also in humans)[13-15] through ligation with ADM-receptors present on vascular smooth muscle cells[6]. Interestingly, complete blockade of ADM signaling in models of murine sepsis or inflammation does not appear to improve survival, although blood pressure was briefly enhanced[16-18]. Currently, it remains unclear whether the increased levels of ADM observed during sepsis are detrimental for the patient due to excessive vasodilation (and hypotension), or whether they represent a failing protective response.

Attempts have been made to pharmacologically modulate ADM's effects in order to improve outcome in sepsis. To this extent, different antibodies (targeting different epitopes of ADM) were administered during murine septic shock and effects on survival were observed for an administered dose of 2 mg/kg[18]. Interestingly, a C-terminal binding antibody that completely blocked ADM signaling did not improve survival, whereas a non-neutralizing antibody that binds to ADM's N-terminal epitope did improve survival (administered as a single i.v. dose of 2 mg/kg). Other work with this non-neutralizing N-terminal antibody (of which the humanized version was eventually named

Adrecizumab) in preclinical models of systemic inflammation and septic shock showed attenuated tissue edema formation and vasopressor requirements, while renal function and survival were improved[19,20]. This consistently occurred for doses of around 2 mg/kg. The proposed mechanism of action relies on an immediate and significant increase of ADM concentrations in the blood upon administration of the antibody, increasing ADM's endothelial stabilizing effects on endothelial cells while reducing interstitial ADM that causes vasodilation[18]. This is further elaborated on in the discussion section. In previously published preclinical work, no safety issues were observed (highest dose tested was 30 mg/kg, in healthy animals)[18]. In human phase I studies (recently published but performed after preclinical safety studies, including those described in the present manuscript), doses of 0.5, 2 and 8 mg/kg were tested and found to be safe[21]. In the present work, we evaluated the toxicological profile, effects on blood pressure, as well as toxicokinetics of Adrecizumab in rodents, dogs and non-human primates.

Methods

Animals

Animal experiments were performed according to local applicable guidelines and regulations (see supplementary materials for a detailed overview). All animals were kept under routine laboratory conditions, with temperature and humidity control, as well as 12h/12h light-dark cycle conditions. Animals were weighed individually and treatment was prepared for individual animals.

The repeated-dose toxicology and toxicokinetics study in rats was performed by ATRC Aurigon Toxicological Research Center Ltd. (Dunakeszi, Hungary). Healthy, naïve, Wistar rats (7-8 weeks old) were obtained from Charles River Laboratories (Sulzfeld, Germany). Rats were fed ad libitum with standard chow (Ssniff R-Z [Ssniff Spezialdiäten GmbH, Soest, Germany]) and water. Rats were kept in polycarbonate cages equipped with metal-wire covers and self-feeding baskets (5 rats/cage during acclimatization, 2-3 rats/cage during the study period and 5 rats/cage during the recovery period in recovery animals).

The repeated-dose cardiovascular and toxicological study in telemetered, conscious, beagle dogs was also performed by ATRC Aurigon Toxicological Research Center Ltd. (Dunakeszi, Hungary). Three healthy female Beagle dogs (24-26 months) were obtained from Marshall BioResources (North Rose, NY, U.S.A). Dogs were quarantined and habituated to laboratory

conditions for at least 5 days. The dogs were housed individually (one dog/pen: 1.2 m2 floor area). Sniff Hd-H diet (ssniff, Spezialdiäten GmbH, Soest, Germany) was offered daily (300 gram/dog) and dogs had unlimited access to water. The dogs were fasted for at least 12 hours before the treatments. On the treatment days, food was distributed approximately 4 hours after treatment administration.

The repeated-dose toxicology and toxicokinetics study in cynomolgus monkeys was performed by SNBL USA, Ltd. (Everett, WA, USA). Purpose-bred, healthy, naïve, cynomolgus monkeys (2-6 years old) were supplied by Tian Hu Cambodia Animal Breeding Research Center or SNBL USA SRC (Alice, Texas, USA) and originated from Cambodia. Animals were housed in cages that comply with the Animal Welfare Act and recommendations set forth in the Guide for the Care and Use of Laboratory Animals (National Research Council 2011). Monkeys were offered PMI's LabDiet® Laboratory Fiber-Plus® biscuits twice a day and drinking water was provided ad libitum. In addition, animals were given fruits and vegetables. An overview of the experiments is provided in **Table 1**. These studies are reported according to ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines.

Ethical approval

Study protocol approval was obtained from the appropriate governing institutes: PEI/001/4557-4/2014 for the rat study (ATRC Animal Ethics Committee), XIV-I-001/288-4/2012 for the beagle dog study (ATRC Animal Ethics Committee), and 78615-02 for the cynomolgus monkey study (IACUC at SNBL USA).

Study medication

Generation of the murine monoclonal antibody HAM1101 directed against the N-terminus of Adrenomedullin has been described previously[18,19]. A humanized recombinant monoclonal antibody (HAM8101;IgG1) was generated from this murine monoclonal antibody by CDR grafting and produced in Chinese hamster ovary cells. HAM8101 was produced by Glycotope Biotechnology GmbH (Heidelberg, Germany) under Good Manufacturing Practice (GMP) conditions, and this antibody is known under the name Adrecizumab. The antibody was kindly provided by Adrenomed AG (Hennigsdorf, Germany) in stock solutions in PBS and were stored at 2-8 °C under temperature-controlled and restricted access conditions. Vehicle solution consisted of 20 mM L-histidine monohydrochloride monohydrate

Table 1. Overview of animal studies.

Study	Purpose	Animal species and total number	Dosage(s) tested	Route of administration
Repeated-dose toxicology and toxicokinetics in rats	14-day toxicology	Wistar rats (n=80)	Vehicle (n=20) 100 mg/kg/day (n=20) 200 mg/kg/day (n=20) 400 mg/kg/day (n=20)	I.v. repeated-dose via tail vein on day 1, 4, 8 and 14
	14-day toxicology with 28-day recovery period	Wistar rats (n=20)	Vehicle (n=10) 400 mg/kg/day (n=10)	
	Toxicokinetic satellite animals	Wistar rats (n=60)	Vehicle (n=6) 100 mg/kg/day (n=18) 200 mg/kg/day (n=18) 400 mg/kg/day (n=18)	
Repeated-dose toxicology in beagle dogs	14-day toxicology with 21-day recovery period	Beagle dogs (n=3)	Vehicle (day 1) 2 mg/kg (day 3) 10 mg/kg (day 5 and 29) 50 mg/kg (day 8)	I.v. via vena cephalica antebrachii
Repeated-dose toxicology and toxicokinetics in cynomolgus monkeys	14-day toxicology	Cynomolgus monkeys (n=24)	Vehicle (n=6) 25 mg/kg/day (n=6) 50 mg/kg/day (n=6) 100 mg/kg/day (n=6)	I.v. via cephalic vein, repeated-dose on day 1, 4, 8 and 14
	14-day toxicology with 28-day recovery period	Cynomolgus monkeys (n=8)	Vehicle (n=4) 100 mg/kg/day (n=4)	

(Sigma, Munich, Germany for rats; JT Baker #K21603 for non-human primates) in water for injection (Teva Pharmaceuticals Works PLtd. Co., Petach Tikva, Israel), pH 6.0. For all groups of rats that were administered Adrecizumab, the drug was diluted with vehicle solution (in a vehicle to Adrecizumab ratio of 3:1 and 1:1 for the 100 mg/kg and 200 mg/kg doses, respectively), except for the 400 mg/kg/day dose (which was ready to use). The study drugs were applied in a volume of 20 mL/kg.

Repeated-dose toxicology and toxicokinetics in rats

Previous, less extensive studies (summarized in the **Supplementary Methods**) were performed to assess acute toxicity (rat pilot study 1 and 2), as well as repeated dose toxicity (rat pilot study 3). Briefly, acute toxicity of Adrecizumab was tested up to 800 mg/kg, a dose which was found to be safe. In the repeated dose study with administration of up to 400 mg/kg Adrecizumab

(via the jugular vein) on 4 consecutive days, 4 of 79 animals showed serious deterioration of physical condition, 2 of which were sacrificed. Due to the fact that there was no dose-dependent relationship and that deterioration also occurred in one of the control group animals, these effects were deemed unlikely related to Adrecizumab administration. Because permanent cannulation of the jugular vein may have attributed to the observed effects, the decision was taken to use tail vein catherization for future studies. In the present work, to assess early (14-day) toxicology, 80 healthy Wistar rats were randomly allocated to 4 groups (n=20 each, divided equally by sex) to receive either vehicle or 100, 200 or 400 mg/kg Adrecizumab by i.v. administration over a 1-hour period via the tail vein on days 1, 4, 8 and 14. To assess late toxicology, 20 additional 'recovery animals' received either vehicle or 400 mg/kg Adrecizumab on the same days (n=10 each, divided equally by sex) and were followed-up for an additional period of 28 days after the last infusion for a total of 42 days. Finally, for determination of toxicokinetics, 60 satellite animals were randomly assigned to 4 groups (divided equally by sex) to receive either vehicle (n=6) or 100, 200 or 400 mg/kg Adrecizumab (n=18 each).

Please refer to **Figure 1** for a detailed overview of the study procedures and endpoints. All animals were observed for general signs of illness, appearance, behavior, mental status, body weight, food consumption, appearance of urine and stools, genitals, and death. Additional observations in early toxicology groups (5 animals of the vehicle control group and 5 animals of the 400 mg/kg group) included the modified Irwin test. Ophthalmological evaluation using split microscopy (PanOptic Ophthalmoscope, Welch Allyn, Skaneateles Falls, NY, USA) was performed in both early toxicology and recovery animals. Urine analysis included visual inspection, microscopic examination of urine sediment and automated analysis with test strips (Medi-Test URYXXON Stick 10) and the URYXXON® 300 analyzer (Machery-Nagel GmbH & Co. KG, Düren, Germany). Blood analyses included parameters of hematology, blood coagulation and biochemistry using a Sysmex XT-200-iV analyzer (Sysmex Corporation, Kobe, Japan), AMAX Destiny Plus coagulator (Trinity Biotech Plc, Bray, Ireland) and Konelab 60i analyzer (Thermo Fischer Scientific, Waltham, Ma, USA), respectively. Differential white blood cell evaluation was done with light microscopy if automated determination was not feasible. Plasma lymphocyte (sub)populations were phenotyped in the short-term (14-day) toxicology animals using flow cytometry (BD FACSCalibur, Beckton Dickinson, Franklin Lakes, NJ, USA). Plasma cytokine analysis was performed

in the vehicle and 400mg/kg/day 14-day toxicology animals only, using a Rat Cytokine 10-Plex Panel Assay Kit (Invitrogen, Carsbad, CA, USA) on a xMAP® system (Luminex, Austin, TX, USA). Animals were sacrificed by rapid exsanguination under deep isoflurane anesthesia on day 15 or 43, followed by macroscopic and histopathological examinations by a pathologist.

Blood collection in toxicokinetic satellite animals was performed at various time points on days 1 and 14 following first and last infusions, respectively, via the retro-orbital plexus (blood from 3 animals per time point, max. 3 samples per animal). Toxicokinetic satellite animals were sacrificed on the day after the last infusion using cervical dislocation under deep isoflurane anesthesia and were not further examined.

Repeated-dose toxicology in telemetered conscious beagle dogs

To assess toxicological and cardiovascular effects of Adrecizumab, 3 female beagle dogs received a single i.v. dose of vehicle on day 1, 2 mg/kg Adrecizumab on day 3, 10 mg/kg Adrecizumab on day 5, 50 mg/kg on day 8, and 10 mg/kg on day 29 (after a 14-day recovery period), which was followed by an additional follow-up period of 22 hours after the last infusion. Treatment was administered via the vena cephalica antebrachii. In order to monitor blood pressure and heart rhythm, a telemetric device was implanted (DSI's telemetry system, with PhysioTel® MultiplusTM implants), which was operated via validated Dataquest A.R.T. software 4.0 (for the recording of all data and analysis of blood pressure).

Please refer to **Figure 2** for a detailed overview of the study procedures and endpoints. Clinical observations were recorded at least twice daily on treatment days and at least once daily on treatment-free days with special regard to behavior, coat, urine and fecal excretion, condition of body orifices and any signs of illness. Measurement of body weight was performed at least once during acclimatization and on each treatment day. Blood pressure (systolic, diastolic and mean) was measured before and at several timepoints after study drug administration on day 1 (vehicle), day 3 (2 mg/kg Adrecizumab), day 5 (10 mg/kg Adrecizumab) and day 8 (50 mg/kg Adrecizumab). In addition, recordings were checked for arrhythmias.

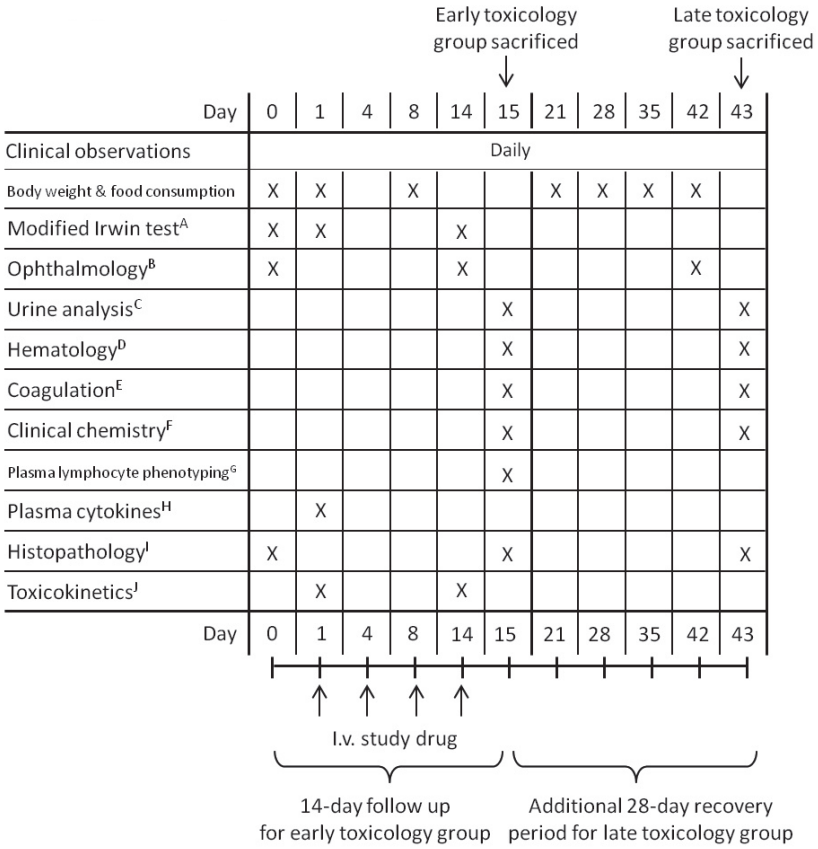


Figure 1. Study procedures of the repeated-dose toxicology and toxicokinetics study in rats. **A.** Only done in the first 5 male and female rats of the vehicle and 400 mg/kg/day group of the early toxicology group, prior to administration and at 3 time points after the first and last infusions (0.5h, 1.5h and 4h). **B.** Examination included cornea, conjunctiva, episcleral vessels, anterior chamber, pupil, lens, retina and optic disc. **C.** Leukocytes, nitrite, pH, glucose, urobilinogen, bilirubin, ketones, erythrocytes, protein, specific gravity, visual inspection, sediment (microscopic) and volume. **D.** Leukocytes and differential leukocyte count, erythrocytes, hemoglobin, hematocrit, mean corpsular volume, mean corpsular hemoglobin, mean corpsular hemoglobin concentration, platelets and reticulocytes. **E.** Prothrombin time and activated partial thromboplastin time. **F.** Alanine aminotransferase activity, aspartate aminotransferase activity, alkaline phosphatase, bilirubin, creatinine, urea, glucose, cholesterol, triglycerides, inorganic phosphates, calcium, sodium, potassium, chloride, albumin, total protein and albumin/globulin ratio. **G.** Total T lymphocytes (CD3+), T-helper (CD3+, CD4+), T-cytotoxic (CD3+, CD8a+), NK-T cells (CD3+, NKR-P1A+), T-activated (CD3+, CD25+), NK cells (CD3-, NKR-P1A+) and B lymphocytes (CD45RA+). **H.** IL-1 beta, IL-2, IL-4, IL-6, IL-10, IL-12, TNF-alpha, IFN-gamma, GM-CSF. Only done in vehicle treated control and 400 mg/kg/day animals, 8 hours after the first study drug administration. Blood was drawn from the retro-orbital plexus. **I.** A complete necropsy was performed with macro- and microscopic evaluation. **J.** Satellite groups of animals were included for toxicokinetics. Blood was drawn from a total of 9 time-points (pre-value, 5m, 15m, 30m, 1h, 2h, 4h, 8h and 24h) after the first and last administrations. At each time point, blood was collected from 3 animals per group with a maximum of 3 samples per animal. Blood was collected from the retro-orbital plexus.

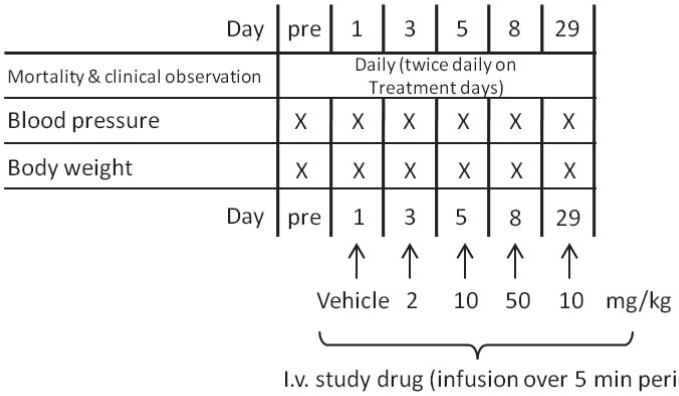


Figure 2. Study procedures of the repeated-dose toxicology in telemetered conscious dogs.

Repeated-dose toxicology and toxicokinetics in cynomolgus monkeys

A previous, less extensive study in 4 animals (summarized in Supplementary Methods) was performed to assess acute toxicity after a single dose of 50 mg/kg (n=1) and 100 mg/kg (n=1), as well as repeated-dose toxicity of 100 mg/kg on 2 days (in n=2 animals). No mortality or other adverse effects were observed for all tested doses. In the present work, the toxicity and toxicokinetics of repeated Adrecizumab administration on separate days over a 14 day period were investigated in cynomolgus monkeys (see **Table 1** for the study design and **Figure 3** for an overview of study procedures and outcomes). To assess early toxicology, 12 male (2 to 4 years old) and 12 female (2 to 6 years old) naïve cynomolgus monkeys were randomly (in a weight-stratified manner) assigned to receive repeated-dose infusion of vehicle or Adrecizumab (25, 50 or 100 mg/kg/day, n=6 each, divided equally by sex) via a peripheral vein on days 1, 4, 8 and 14. To assess late toxicology, 8 additional 'recovery animals' received either vehicle or 100 mg/kg Adrecizumab on the same days (n=4 each, divided equally by sex) and were followed-up for an additional period of 28 days after the last infusion for a total of 42 days. Animals were acclimated to the study room for 14 days prior to dosing on day 1.

The following parameters were assessed: Mortality and clinical observation, body weight, food consumption (per social group of animals), neurological observations, blood pressure (collected under ketamine sedation by BAS Vetronics – Vital Scan Monitor), respiratory rate (under ketamine sedation), electrocardiography (under ketamine sedation, using Ponemah Physiology Platform Software, Version 5.2, Data Services International), ophthalmology (under ketamine sedation, using indirect ophthalmoscope and slit lamp

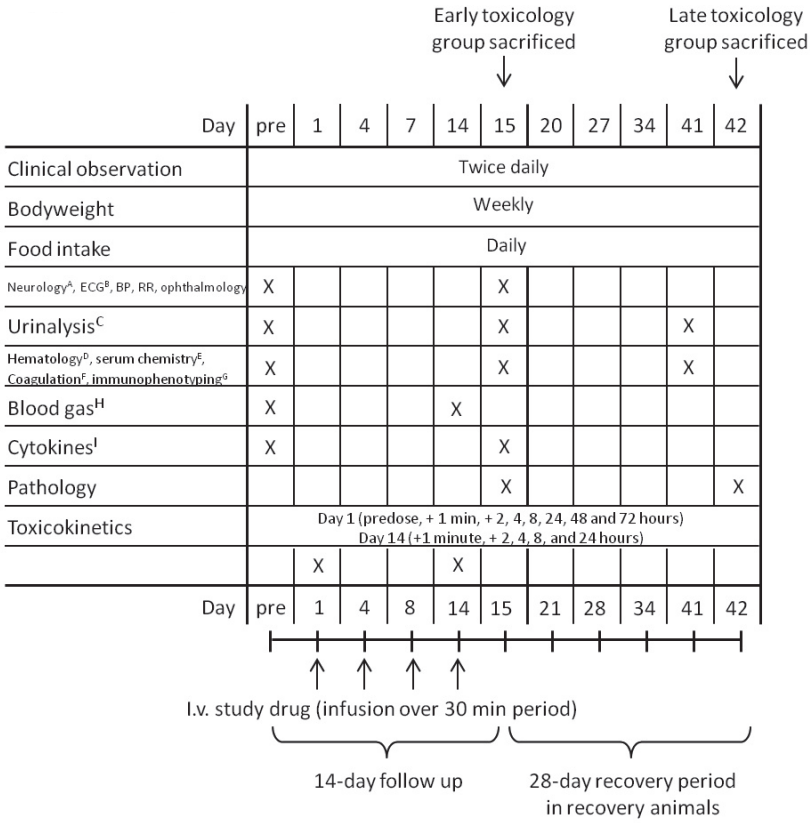


Figure 3. Study procedures of the repeated-dose toxicology and toxicokinetics study in cynomolgus monkeys. **A.** Neurological examination included stimulus response, behavior, gait, brachiation, posture, grasp, activity level, balance, conjugate movement, position and pupil reactivity. **B.** Electrocardiography (ECG) included RR and PR interval, QRS duration, QT interval and Bazett's corrected QT interval (QTc), heart rate (HR), measured over a time period of at least 5 consecutive cardiac beats at each time point. **C.** Urine chemistry included bilirubin, clarity, color, glucose, ketones, leukocytes, nitrites, occult blood, pH, protein, specific gravity, urobilinogen, volume, sodium, potassium, chloride and creatinine. **D.** Hematological parameters included hematocrit, hemoglobin, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, mean platelet volume, platelets, red blood cells, red cell distribution width, reticulocytes absolute & percent, white blood cells and differential leukocyte count (absolute). **E.** Serum chemistry included alanine aminotransferase, albumin, albumin/globulin ratio, alkaline phosphatase, aspartate aminotransferase, blood urea nitrogen, calcium, chloride, creatine kinase, creatinine, gamma glutamyltransferase, globulin, glucose, inorganic phosphorus, potassium, sodium, total bilirubin, total cholesterol, total protein, triglyceride, IgG. **F.** Coagulation included activated partial thromboplastin time, fibrinogen and prothrombin time (PT). **G.** Flow cytometric lymphocyte phenotyping was conducted on isolated leukocytes using antibodies to CD3, CD4, CD8, CD16, CD20, CD25, and CD69. Populations included total T cells, T helper cells, T cytotoxic cells, regulatory T helper cells, natural killer (NK) cells, B cells, activated T cells, activated NK cells and activated B cells. **H.** Blood gas analyses included lactate, pH, PCO2, PO2, TCO2, HCO3, Base Excess, sO2 (oxygen saturation). **I.** Cytokines were determined in the 100 mg/kg/day group only and included IL (interleukin)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFNγ (interferon gamma) and TNFα (tumor necrosis factor alpha).

microscopy, with topical administration of mydriatics). Clinical pathology included urinalysis (collected using urine pans placed beneath animal cages on the evening before collection, analyzed using Clinitek Advantus, AU680 automated analyzer, Sper Refractometer and manual/visual inspection), hematology (1.3 mL blood collected in K2EDTA-containing tubes, analyzed using Advia 120 automated analyzer or manually using an Olympus microscope), coagulation (1.8 mL blood in 3.2% sodium citrate-containing tubes, analyzed with a STACompact automated analyzer), serum chemistry (1 mL blood in serum separator tubes, analyzed using an AU 680 analyzer) and blood gas (0.3 mL blood analyzed using a Marquest™ Gaslyte® syringe system from the femoral artery or tail artery), immunoglobulin E (IgE, 0.5 mL blood in serum separator tubes, analyzed by ANTECH GLP using an ELISA method developed and validated by ANTECH GLP), lymphocyte phenotyping (1-2 mL blood collected in K3EDTA-containing tubes, analyzed in a FACSCanto™ II flow cytometer running FACSDiva™ acquisition software [version 6.1.3]), and cytokines (1 mL blood collected in serum separator tubes). Serial blood samples for toxicokinetics were collected at specified timepoints on days 1 and 14. Animals were sacrificed on day 15 (early toxicity) or 42 (recovery animals) by intravenous injection of Euthanasol, a commercially available euthanasia solution while sedated, followed by exsanguination, macroscopic and histopathological examinations by a pathologist.

Toxicokinetic evaluation in rats and monkeys

Approximately 0.5 mL was collected in K2EDTA-containing tubes and stored below -60 to -86 °C until batchwise analyses. The toxicokinetic analysis was performed by ATRC Aurigon Toxicological Research Center Ltd. (Dunakeszi, Hungary) using validated Phoenix WinNonlin Version 6.3 (Pharsight Corporation, USA). Unbound Adrecizumab was quantified in the samples using a validated luminescence immunoassay with a quantification limit of 0.797 µg/mL. Plasma concentration-time curves were evaluated using non compartmental method. The following parameters were calculated from the measured data: Maximum observed plasma concentration (Cmax), last quantifiable concentration (Clast), time of maximum observed plasma concentration (Tmax), time at last quantifiable concentration (Tlast), terminal elimination half-life (t1/2), dose normalized Cmax (Cmax/dose), area under the plasma concentration-time curve from time zero up to 24 hours (AUC0-24h) and dose normalized AUC0-24h (AUC0-24h/dose). AUC values were calculated by linear trapezoidal integration. The accumulation ratio Racc was calculated from the mean AUC0-24h values as follows: Racc = AUC0-24h on

day 14 / AUC0-24h on day 1. In rats, there were not enough data to calculate terminal elimination half-life.

Adrenomedullin measurements

The ADM assay was performed as described previously[7]. As ADM is highly conserved across mammalian species, the same assay was used for both species. Briefly, plasma ADM concentrations (including both free and Adrecizumab-bound ADM) were measured by a sandwich coated luminescence immunoassay, based on acridinium NHS-ester labeling and anti-ADM antibodies (solid phase antibody targeting the mid region of ADM and a labeled antibody against the amidated C-terminal region of ADM). Dilutions of ADM and labeled tracer were subsequently added to antibody-coated wells. After washing of unbound tracer, chemiluminescence was measured and evaluated against a standard curve.

Statistical analysis

Data are presented as mean and standard deviation or as median and interquartile rate depending on its distribution. Statistics were performed using the SAS package for Windows (SAS Institute Inc., Cary, NC, USA), version 6.12. In addition, analyses were performed separately for each sex. Data from early toxicology and recovery animals were combined at each shared scheduled time-point up to the terminal necropsy. A two-tailed p-values <0.05 were considered statistically significant.

For the rat study, the Shapiro-Wilk test was performed to test the normality and homogeneity of the variance of two groups. In case the Shapiro-Wilk test was negative, the F-test was used in order to determine which of the three types of t-tests had to be employed. If the F-test indicated homogenous variances, the Student's t-test was used. If the F-test was significant and the two groups had an equal numbers of data, the Satterthwaite t-test was performed. If the F-test was significant and the number of data within the groups was not equal, the Cochran t-test was used. For non-parametric data, the Wilcoxon rank-sum test was applied. When analyzing more than two groups, the homogeneity of the group variance was analysed using Bartlett's test. In case of homogeneous variances, one-way ANOVA was used, with a Dunnett post-hoc test if the ANOVA was significant. In case of inhomogeneous variances, the Kruskall-Wallis test was used, with non-parametric multiple comparisons (by Gad and Weil) in case the Kruskall-Wallis test was significant. For the beagle dog and cynomolgus monkey studies, no statistical analyses were performed due to small group sizes.

Results

Repeated-dose toxicology and toxicokinetics in rats

Mortality and clinical findings

Repeated intravenous infusion of Adrecizumab on 4 separate days at doses of 100, 200, and 400 mg/kg body weight per day did not result in mortality. The most notable observed clinical sign was a red discoloration of urine (**Table S1**), which could be observed in all vehicle-treated animals on all 4 infusion days, and in a total of 6 animals of the 100 mg/kg/day dose group on the first infusion day only. This finding was not observed in the 200 and 400 mg/kg/day groups, and was attributed to the vehicle (further elaborated on in the discussion). The only other clinical finding was observed in 1 male rat from the 200 mg/kg/day dose toxicokinetics group (**Table S1C**), and was considered to be unrelated to Adrecizumab. The rat showed decreased activity and body weight, porphyrin around nose and eyes, piloerection, pale mucosa and skin from day 8 to day 14.

Body weight and food intake

Body weight of all rats increased normally throughout the study period and was statistically (but not relevantly) different only for male rats treated with 100 mg/kg Adrecizumab compared to vehicle treated rats (**Figure 4A**). Food consumption was also not significantly affected by Adrecizumab (**Figure 4B**).

Modified Irwin test

Sensory reactivity to different stimuli (auditory, visual and proprioceptive), as well as qualitative assessment of grip strength and motor activity in the high dose animals (400 mg/kg/day) did not show any deviation from the vehicle group (data not shown). No significant differences were observed in body temperature (data not shown).

Ophthalmology

Ophthalmological examination using a slit light ophthalmoscope revealed no abnormalities at baseline, 14 and 42 days after the first study drug infusion (**Table S2**).

Hematology

In male rats, RBC and MCV were statistically significantly increased in the 200 and 400 mg/kg/day dose groups (**Table 2**). Hb, Ht and absolute lymphocyte numbers were significantly increased in the 400 mg/kg/day dose

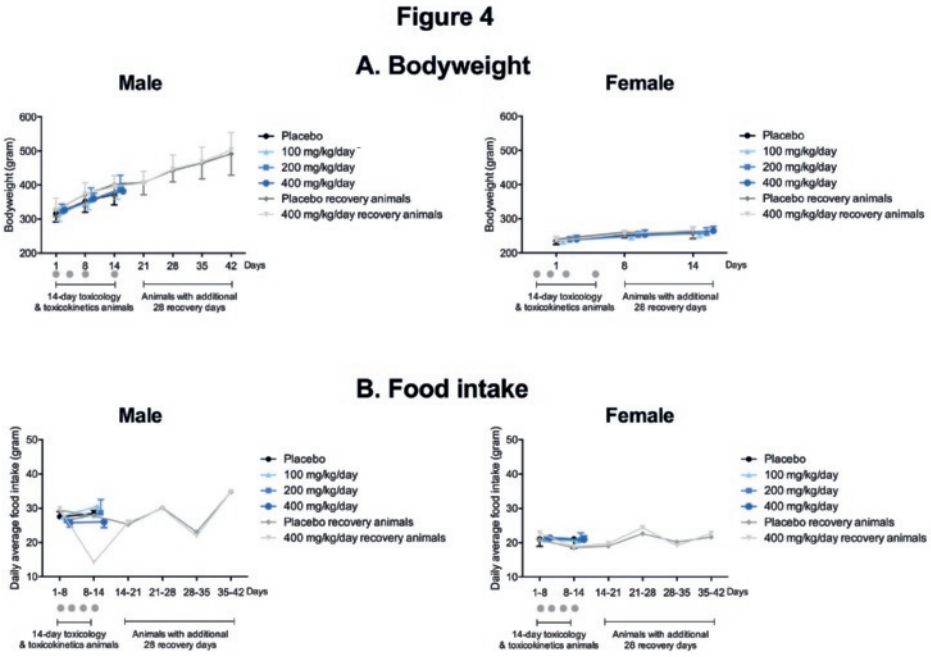


Figure 4. Bodyweight and food consumption in rats. Data are expressed as mean ± SD. Grey dots indicate days on which study drug was administered (day 1, 4, 8 and 14).

group. Absolute and relative numbers of reticulocytes were significantly lower in all Adrecizumab groups. In female rats, RBC, Hb and Ht were significantly increased in all Adrecizumab groups, and MCV, MCH, as well as MCHC were significantly increased in 100 and 200 mg/kg/day dose groups (**Table 2**). The absolute numbers of reticulocytes were significantly increased in 200 and 400 mg/kg/day dose groups, and the relative number of reticulocytes were significantly increased in all Adrecizumab groups. In the 400 mg/kg/day female recovery animals, RBC and HT were significantly increased on day 43. All values were within the normal range and therefore considered toxicologically irrelevant.

Table 2. Hematology in rats.

Day	Parameter	Male				Female			
		Vehicle	100 mg/kg /day	200 mg/kg /day	400 mg/kg /day	Vehicle	100 mg/kg/day	200 mg/kg/day	400 mg/kg/day
Day 15	No. of animals	n=10	n=10	n=10	n=10	n=10	n=10	n=10	n=10
	WBC (10^9/L)	9.6 ± 2.11	7.9 ± 0.97	9.4 ± 1.37	11.2 ± 1.86	5.8 ± 1.49	5.4 ± 0.86	6.0 ± 1.40	5.4 ± 1.82
	RBC (10^{12}/L)	7.4 ± 0.49	7.9 ± 0.42	8.3 ± 0.34*	8.5 ± 0.57*	6.8 ± 0.45	7.8 ± 0.48*	7.8 ± 0.41*	7.5 ± 0.41*
	Hb (g/L)	147.9 ± 8.61	150.9 ± 5.45	156.5 ± 8.77	162.7 ± 9.52*	133.0 ± 5.87	144.8 ± 6.01*	143.4 ± 8.07*	143.4 ± 7.31*
	Ht (L/L)	0.4 ± 0.02	0.4 ± 0.01	0.4 ± 0.02	0.5 ± 0.03*	0.4 ± 0.01	0.4 ± 0.01*	0.4 ± 0.02*	0.4 ± 0.02*
	MCV (fL)	56.4 ± 3.47	53.9 ± 2.88	53.1 ± 2.24*	53.1 ± 1.69*	55.3 ± 2.49	52.0 ± 2.25*	51.1 ± 1.60*	53.6 ± 2.00
	MCH (pg)	19.9 ± 0.89	19.2 ± 0.83	19.0 ± 0.72*	19.1 ± 0.60	19.5 ± 0.77	18.7 ± 0.64*	18.5 ± 0.52*	19.0 ± 0.49
	MCHC (g/L)	353.2 ± 9.10	356.0 ± 6.04	357.5 ± 4.81	359.5 ± 5.84	352.5 ± 6.40	359.9 ± 5.13*	361.7 ± 3.06*	356.1 ± 6.14
	PLT (10^9/L)	1013.6 ± 133.26	930.2 ± 210.01	1006.0 ± 124.87	1114.9 ± 134.10	966.1 ± 240.30	1129.0 ± 170.14	1057.8 ± 137.50	1023.6 ± 139.07
	Ret (10^9/L)	479.7 ± 64.38	384.3 ± 66.41*	369.1 ± 37.74*	397.7 ± 69.44*	411.6 ± 68.50	365.0 ± 61.29	311.9 ± 54.40*	277.0 ± 52.50*
	Ret (%)	6.5 ± 0.90	4.9 ± 0.78*	4.5 ± 0.38*	4.7 ± 0.92*	6.1 ± 1.14	4.7 ± 0.88*	4.0 ± 0.67*	3.7 ± 0.82*
	Neut (10^9/L)	2.2 ± 1.31	1.7 ± 1.01	1.7 ± 0.92	2.1 ± 1.11	1.4 ± 0.75	1.0 ± 0.29	1.3 ± 0.50	1.3 ± 0.90
	Lymph (10^9/L)	6.7 ± 1.54	5.6 ± 1.05	7.1 ± 1.87	8.4 ± 1.59*	4.0 ± 1.14	4.0 ± 0.71	4.2 ± 1.35	3.7 ± 1.12
	Mono (10^9/L)	0.5 ± 0.16	0.4 ± 0.11	0.5 ± 0.10	0.5 ± 0.19	0.3 ± 0.12	0.3 ± 0.10	0.3 ± 0.13	0.3 ± 0.15
	Eo (10^9/L)	0.1 ± 0.05	0.1 ± 0.11	0.1 ± 0.09	0.1 ± 0.05	0.2 ± 0.07	0.1 ± 0.04	0.1 ± 0.06	0.1 ± 0.06
	Baso (10^9/L)	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
	Neut (%)	22.8 ± 9.21	21.8 ± 11.17	18.6 ± 11.22	18.4 ± 7.74	22.9 ± 8.86	18.1 ± 5.11	23.0 ± 8.89	24.1 ± 8.65
	Lymph (%)	70.3 ± 9.24	71.4 ± 10.89	74.7 ± 12.31	75.6 ± 8.89	69.2 ± 9.71	74.5 ± 5.27	70.1 ± 9.58	68.5 ± 8.81
	Mono (%)	5.3 ± 1.40	4.9 ± 1.23	5.1 ± 0.97	4.8 ± 1.38	4.6 ± 1.50	4.9 ± 1.10	4.6 ± 1.99	5.1 ± 1.89
	Eo (%)	1.5 ± 0.58	1.8 ± 1.28	1.6 ± 1.15	1.2 ± 0.61	3.3 ± 1.25	2.4 ± 0.76	2.3 ± 0.94	2.3 ± 0.94
	Baso (%)	0.1 ± 0.05	0.1 ± 0.06	0.1 ± 0.05	0.1 ± 0.00	0.0 ± 0.07	0.0 ± 0.08	0.0 ± 0.07	0.1 ± 0.08

Table 2. continued.

Day	Parameter	Male				Female			
		Vehicle	100 mg/kg /day	200 mg/kg /day	400 mg/kg /day	Vehicle	100 mg/kg/day	200 mg/kg/day	400 mg/kg/day
Day 42 (recovery animals)	No. of animals	n=5	n=0	n=0	n=5	n=5	n=0	n=0	n=5
	WBC (10^9/L)	10.0 ± 3.80			13.8 ± 3.04	9.6 ± 2.35			7.5 ± 0.81
	RBC (10^{12}/L)	8.5 ± 0.71			8.8 ± 0.51	7.6 ± 0.44			8.3 ± 0.24*
	Hb (g/L)	156.2 ± 11.32			163.2 ± 4.32	142.2 ± 8.76			155.0 ± 9.35
	Ht (L/L)	0.4 ± 0.03			0.4 ± 0.01	0.4 ± 0.02			0.4 ± 0.02*
	MCV (fL)	50.7 ± 2.84			51.2 ± 2.66	51.8 ± 1.34			51.7 ± 2.72
	MCH (pg)	18.5 ± 0.71			18.7 ± 0.78	18.7 ± 0.38			18.7 ± 1.05
	MCHC (g/L)	365.6 ± 7.64			364.8 ± 6.50	361.6 ± 4.28			362.6 ± 4.77
	PLT (10^9/L)	1064.6 ± 83.03			1008.6 ± 165.05	884.2 ± 59.61			1025.0 ± 126.64
	Ret (10^9/L)	269.8 ± 67.57			309.5 ± 39.28	191.2 ± 23.50			212.8 ± 25.54
	Ret (%)	3.2 ± 0.81			3.5 ± 0.40	2.5 ± 0.30			2.6 ± 0.34
	Neut (10^9/L)	1.8 ± 0.57			1.6 ± 0.39	1.6 ± 0.45			1.2 ± 0.26
	Lymph (10^9/L)	7.5 ± 3.42			11.3 ± 3.15	7.3 ± 1.90			5.7 ± 0.55
	Mono (10^9/L)	0.5 ± 0.19			0.6 ± 0.15	0.4 ± 0.09			0.3 ± 0.10
	Eo (10^9/L)	0.2 ± 0.08			0.2 ± 0.09	0.2 ± 0.08			0.2 ± 0.06
	Baso (10^9/L)	0.0 ± 0.01			0.0 ± 0.01	0.0 ± 0.00			0.0 ± 0.00*
	Neut (%)	21.2 ± 12.08			12.3 ± 3.97	17.0 ± 3.44			16.2 ± 2.25
	Lymph (%)	71.7 ± 12.37			81.6 ± 5.92	76.5 ± 4.12			76.9 ± 3.46
	Mono (%)	5.3 ± 0.64			4.6 ± 1.48	4.0 ± 0.92			4.1 ± 1.19
	Eo (%)	1.8 ± 0.45			1.5 ± 0.73	2.4 ± 0.47			2.8 ± 0.88
	Baso (%)	0.1 ± 0.05			0.1 ± 0.04	0.1 ± 0.04			0.0 ± 0.00*

Data are expressed as mean ± SD.* $p \leq 0.05$ compared to vehicle. *Abbreviations: hematocrit (Ht), hemoglobin (Hb), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean platelet volume (MPV), platelets, red blood cells (RBC), red cell distribution width (RBCW), reticulocytes absolute & percentual, white blood cells (WBC) and differential leukocyte count (absolute).*

Clinical chemistry and coagulation

In both male and female rats treated with Adrecizumab, significantly higher total protein concentrations in plasma were observed (**Table 3**). In addition, a (dose-dependent) decrease of the albumin/globulin ratio (A/G) indicated that the increase of total protein concentrations was due increased levels of (immuno)globulins, since albumin remained unchanged. In a few cases, statistical analysis showed significant changes in other parameters (e.g. ALT and AST in males and ALT in females on day 15), although these differences between the vehicle and Adrecizumab groups were caused by the relatively lower values of the vehicle animals and not by increased values in Adrecizumab-treated animals. All values were within the normal range and therefore considered toxicologically irrelevant.

Coagulation parameters were not relevantly influenced by Adrecizumab (**Table 3**). All parameters were within the normal range of variation, and only one significant difference was observed (PT: 22 ± 0.6 seconds in the vehicle group versus 21 ± 0.7 and 21 ± 0.7 seconds in the 200 and 400 mg/kg/day dose groups, respectively; both $p<0.05$).

Urinalysis

Urine parameters were not relevantly influenced by Adrecizumab (**Table S3**). A slightly lower volume of urine and higher specific gravity was observed in Adrecizumab-treated male rats on day 15, and a slightly higher specific gravity was also observed in 400 mg/kg/day treated female rats. However, all findings were within the normal range of variance for the species and therefore not considered toxicologically relevant.

Plasma lymphocyte phenotyping and plasma cytokines

Neither lymphocyte subpopulations (**Table S4**), nor plasma cytokine concentrations (**Table S5**) were relevantly influenced by Adrecizumab. Note that most cytokines remained below the quantification limit, and therefore did not relevantly increase. Concentrations within the quantification limits were only observed for IL-2 and IL-12 and did not differ between groups.

Necropsy and histopathology

Necropsies with macroscopic observation, weighing of organs and histopathological examination by a pathologist were performed in toxicological animals (animals were sacrificed on day 15 or day 43). All macroscopic findings were considered unrelated to Adrecizumab (**Table S6**). Red areas

were observed in the lungs of both all groups, including control animals, but considered to be the consequences of deep anesthesia (since animals were sacrificed by induction of deep isoflurane anesthesia, followed by rapid exsanguination). Organ weight (absolute and relative) did only differ in a few cases which were considered incidental and without biological relevance (**Table S7**).

Histopathological examination did not reveal any effects that were considered related to Adrecizumab treatment (**Table S8**). The microscopic abnormalities that were observed at the site of administration were generally considered to be procedural in origin. However, a local irritative effect caused by the treatment could not be fully excluded because blood clot or fibrin precipitate was seen in the vein lumen of animals treated with 200 and 400 mg/kg/day, and because recanalisation of organized vein lumen was observed in one 400 mg/kg/day recovery animal.

Toxicokinetics

Plasma Adrecizumab concentration time-profiles of male and female rats are depicted in Figure 5, and toxicokinetic parameters are listed in Table S9. None of the samples collected from vehicle-treated animals contained detectable Adrecizumab concentrations, indicating that there was no cross-contamination between the vehicle and Adrecizumab-treated groups (vehicle group data not shown). In all three dose groups, the Cmax was attained almost immediately after termination of infusion. The dose-normalized maximum concentrations (Cmax/dose) and dose-normalized AUC0-24h (AUC0-24h/dose) showed dose-proportionality on day 1, but not after the 4th infusion on day 14. The disappearance of Adrecizumab from the circulation was very slow for all doses (**Figure 5**) The accumulation ratio (Racc) was dose-proportional in the 100 (1.73 and 1.85 for males and females, respectively) and 200 mg/kg/day group (1.97 and 1.60 for males and females, respectively), but slightly sub-proportional in the 400 mg/kg/day group (1.33 and 1.38 for males and females, respectively).

Circulating adrenomedullin concentrations

Administration of Adrecizumab resulted in a rapid and dose-dependent increase of circulating levels of total ADM (the assay detects both free and Adrecizumab-bound ADM; **Supplementary Figure 1)**.

Table 3. Clinical chemistry and coagulation in rats.

Day	Parameter	Male				Female			
		Vehicle	100 mg/kg/day	200 mg/kg/day	400 mg/kg/day	Vehicle	100 mg/kg/day	200 mg/kg/day	400 mg/kg/day
Day 15	No. of animals	n=10	n=10	n=10	n=10	n=10	n=10	n=10	n=10
	ALT (U/L)	38.6 ± 8.52	42.1 ± 10.64	55.4 ± 6.34*	54.0 ± 13.76*	31.6 ± 3.35	40.9 ± 8.97*	43.0 ± 14.56*	46.1 ± 7.36*
	AST (U/L)	138.5 ± 31.93	145.2 ± 48.11	229.6 ± 94.61*	186.7 ± 58.95	126.5 ± 21.99	141.9 ± 40.18	149.5 ± 38.34	149.0 ± 41.82
	ALP (U/L)	119.3 ± 24.48	142.6 ± 28.28	144.9 ± 39.98	134.7 ± 23.69	64.1 ± 14.49	57.6 ± 14.52	59.1 ± 7.95	82.3 ± 35.84
	GGT (U/L)	1.4 ± 0.32	1.6 ± 0.48	1.1 ± 0.35	1.3 ± 0.91	1.6 ± 0.52	1.7 ± 0.43	1.8 ± 0.29	1.7 ± 0.37
	Total bilirubin (µmol/L)	1.6 ± 0.20	1.4 ± 0.18	1.7 ± 0.26	1.2 ± 0.33*	1.5 ± 0.23	1.7 ± 0.24	1.7 ± 0.33	1.8 ± 0.26
	Creatinine (µmol/L)	22.8 ± 2.78	23.4 ± 3.05	23.3 ± 2.32	21.6 ± 3.02	24.6 ± 2.52	25.3 ± 3.14	24.3 ± 1.97	25.0 ± 1.60
	Urea (mmol/L)	5.5 ± 0.87	5.1 ± 0.71	5.3 ± 0.75	6.1 ± 1.13	5.9 ± 1.69	5.8 ± 0.91	5.9 ± 0.82	6.2 ± 1.01
	Glucose (mmol/L)	13.0 ± 2.62	10.3 ± 2.34*	8.9 ± 1.69*	10.2 ± 2.20*	8.6 ± 1.99	8.4 ± 1.56	8.3 ± 1.72	9.3 ± 1.04
	Cholesterol (mmol/L)	1.6 ± 0.32	1.6 ± 0.31	1.4 ± 0.33	1.4 ± 0.33	1.7 ± 0.30	1.6 ± 0.19	1.6 ± 0.44	1.7 ± 0.27
	Triglycerides (mmol/L)	0.6 ± 0.19	0.5 ± 0.12	0.4 ± 0.12	0.6 ± 0.27	0.4 ± 0.08	0.4 ± 0.15	0.4 ± 0.12	0.4 ± 0.12
	Pi (mmol/L)	3.3 ± 0.36	3.2 ± 0.20	3.3 ± 0.36	3.0 ± 0.25	3.0 ± 0.23	3.3 ± 0.27	2.9 ± 0.35	2.9 ± 0.40
	Ca^{2+} (mmol/L)	2.7 ± 0.13	2.7 ± 0.10	2.7 ± 0.11	2.7 ± 0.09	2.7 ± 0.11	2.8 ± 0.07	2.7 ± 0.07	2.7 ± 0.11
	Na^{+} (mmol/L)	138.5 ± 1.35	139.5 ± 1.35	140.3 ± 1.25*	140.3 ± 1.34*	138.6 ± 1.26	139.9 ± 1.60	139.7 ± 1.16	139.1 ± 1.52
	K^{+} (mmol/L)	6.0 ± 1.00	5.7 ± 0.52	5.9 ± 0.73	5.5 ± 0.34	5.3 ± 0.57	5.3 ± 0.44	5.3 ± 0.32	5.1 ± 0.60
	Cl (mmol/L)	102.0 ± 0.88	102.4 ± 1.08	102.6 ± 1.21	102.6 ± 0.75	101.8 ± 1.16	102.6 ± 1.22	102.3 ± 1.01	101.6 ± 1.15
	Albumin (g/L)	30.7 ± 1.21	30.5 ± 1.18	31.6 ± 0.68	30.4 ± 1.70	31.3 ± 2.18	33.3 ± 1.53*	33.1 ± 1.30	31.7 ± 1.50
	Total protein (g/L)	52.0 ± 2.83	54.4 ± 3.17	57.8 ± 2.62*	58.5 ± 3.60*	51.9 ± 2.46	57.4 ± 1.62*	57.6 ± 3.72*	58.2 ± 3.34*
	A/G	1.45 ± 0.14	1.27 ± 0.07*	1.22 ± 0.09*	1.09 ± 0.11	1.54 ± 0.14	1.39 ± 0.09*	1.37 ± 0.13*	1.20 ± 0.08*
	PT (s)	20.3 ± 1.38	20.6 ± 1.33	21.1 ± 1.86	20.6 ± 1.44[A]	22.3 ± 0.64	21.5 ± 1.42	21.2 ± 0.72*	21.3 ± 0.73*
	APTT (s)	21.0 ± 2.99	19.8 ± 3.44	22.2 ± 3.09	19.7 ± 2.24	18.6 ± 1.51	19.3 ± 1.78	18.1 ± 2.06	18.7 ± 2.06

Table 3. continued.

Day	Parameter	Male				Female			
		Vehicle	100 mg/kg/day	200 mg/kg/ day	400 mg/kg/day	Vehicle	100 mg/kg/ day	200 mg/kg/ day	400 mg/kg/ day
Day 42 (recovery animals)	No. of animals	n=5	n=0	n=0	n=5	n=5	n=0	n=0	n=5
	ALT (U/L)	45.2 ± 10.65			33.6 ± 2.37	36.9 ± 11.99			32.5 ± 1.01
	AST (U/L)	143.8 ± 26.92			114.2 ± 8.23	132.2 ± 56.57			107.2 ± 15.72
	ALP (U/L)	57.9 ± 32.25			94.4 ± 15.08	43.8 ± 12.91			62.2 ± 17.30
	GGT (U/L)	0.4 ± 0.13[B]			n.d.	0.3 ± 0.29			0.4 ± 0.22
	Total bilirubin (µmol/L)	1.4 ± 0.12			1.6 ± 0.51	1.9 ± 0.43			2.2 ± 0.63
	Creatinine (µmol/L)	23.1 ± 3.32			23.4 ± 1.25	23.7 ± 1.90			24.4 ± 1.77
	Urea (mmol/L)	4.8 ± 0.24			5.0 ± 0.85	5.3 ± 0.66			5.8 ± 0.32
	Glucose (mmol/L)	8.5 ± 0.96			7.3 ± 1.70	10.0 ± 1.23			10.1 ± 1.21
	Cholesterol (mmol/L)	1.5 ± 0.77			1.3 ± 0.21	2.0 ± 0.15			1.7 ± 0.30
	Triglycerides (mmol/L)	0.4 ± 0.12			0.5 ± 0.18	0.5 ± 0.10			0.5 ± 0.11
	Pi (mmol/L)	2.5 ± 0.14			2.8 ± 0.19*	2.6 ± 0.28			2.2 ± 0.18*
	Ca^{2+} (mmol/L)	2.6 ± 0.08			2.6 ± 0.11	2.7 ± 0.07			2.6 ± 0.06
	Na^{+} (mmol/L)	143.0 ± 1.41			142.6 ± 0.55	140.0 ± 0.71			140.8 ± 1.64
	K^{+} (mmol/L)	4.6 ± 0.16			4.5 ± 0.21	4.4 ± 0.14			4.4 ± 0.38
	Cl (mmol/L)	103.9 ± 2.14			104.1 ± 1.13	101.7 ± 0.47			103.7 ± 1.14*
	Albumin (g/L)	31.3 ± 1.10			32.3 ± 1.00	33.5 ± 0.56			33.1 ± 1.64
	Total protein (g/L)	53.2 ± 2.54			57.4 ± 1.61*	55.7 ± 1.61			56.2 ± 3.12
	A/G	1.44 ± 0.11			1.30 ± 0.00*	1.50 ± 0.14			1.42 ± 0.08
	PT (s)	22.6 ± 1.50			23.2 ± 1.94	22.0 ± 1.50			22.0 ± 1.48
	APTT (s)	19.0 ± 2.48			21.0 ± 3.41	19.5 ± 0.71			17.8 ± 4.83

Data are expressed as mean ± SD. * $p \leq 0.05$ compared to vehicle. [A] Data from 2 animals missing. [B] Data of 1 animal missing. *Abbreviations: Activated partial thromboplastin time (APTT), alanine aminotransferase (ALT), albumin (ALB) albumin/globulin ratio (A/G), alkaline phosphatase (ALP), aspartate aminotransferase (AST), calcium (CA), creatine kinase (CK), creatinine (CRN), gamma glutamyltransferase (GGT), immunoglobulin G (IgG), prothrombin time (PT), (APTT), total bilirubin (TBIL), total cholesterol (TChol), triglyceride (TGC).*

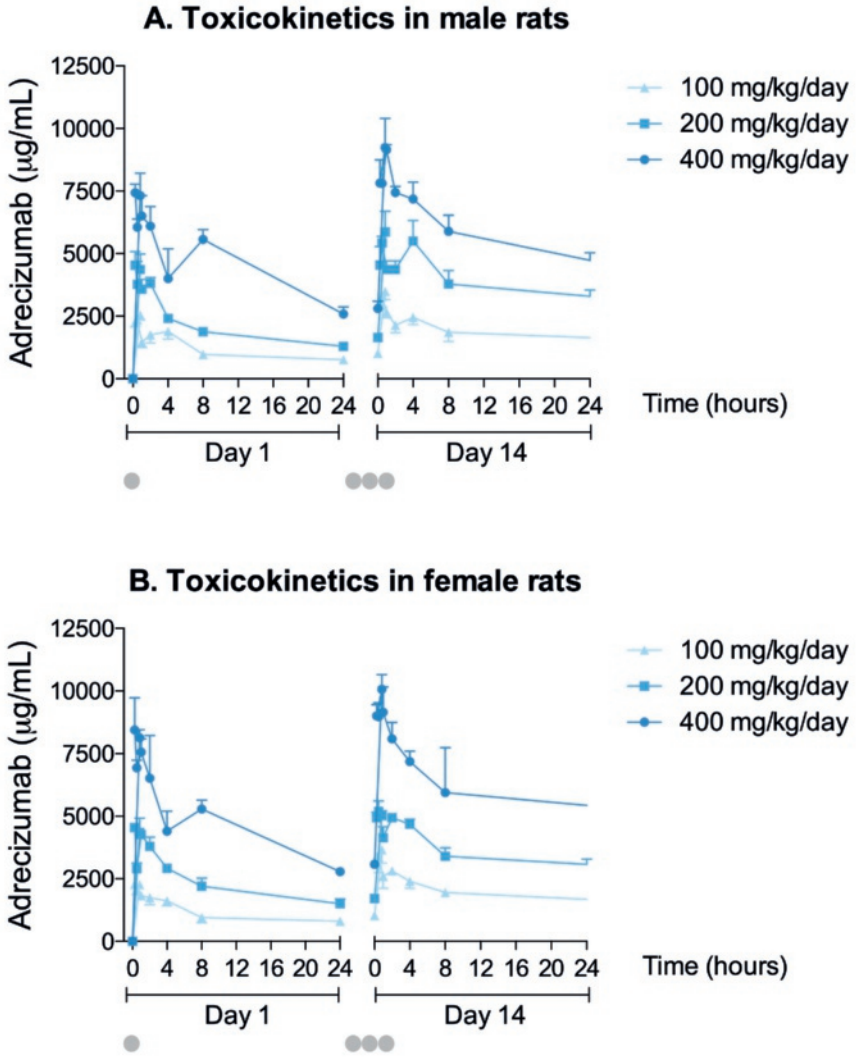


Figure 5. Toxicokinetics in rats. Grey dots indicate days on which study drug was administered (day 1, 4, 8 and 14). Data are expressed as mean ± SD.

Repeated-dose toxicology in telemetered conscious dogs

Mortality and clinical findings

No mortality was observed during this study. Also, no clinical findings were observed after the first four treatment administrations on days 1 (vehicle), 3 (2 mg/kg), 5 (10 mg/kg) and 8 (50 mg/kg). However, after the fifth and final treatment (10 mg/kg on day 29), several transient clinical findings were observed, including weakness (3 animals), moderately decreased activity (2 animals), preference for lying position (2 animals), vomiting (2 animals), spontaneous defecation (2 animals) and spontaneous urination (1 animal). Specifics are detailed in **Table S10**. All animals recovered within 24 hours.

Body weight

Body weight did not appear changed during the course of the study (**Supplementary Figure 2**).

Blood pressure and heart rhythm

There appeared to be no changes in blood pressure parameters (systolic, diastolic and mean blood pressure [MAP]) after any of the treatments (MAP shown in **Figure 6**). No arrhythmias were observed.

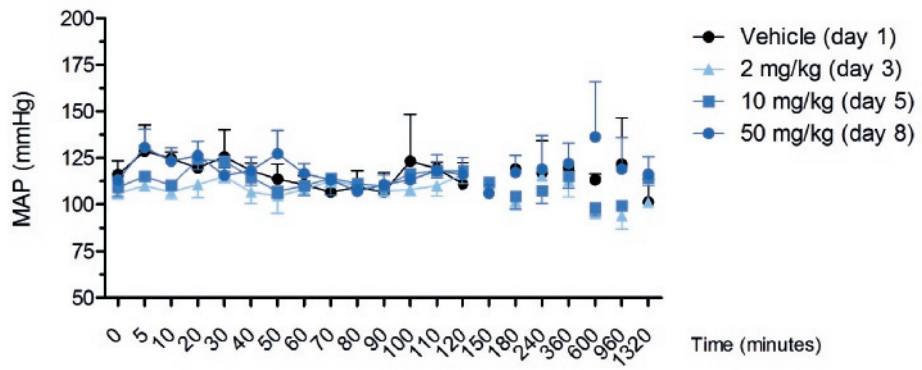


Figure 6. Blood pressure in Beagle dogs. Data are expressed as mean ± SD. The first 22 hours after treatment administration of the 4 respective doses are shown, on day 1, 3, 5 and 8.

Repeated-dose toxicology and toxicokinetics in cynomolgus monkeys

Mortality and clinical findings

Repeated intravenous infusion of Adrecizumab on 4 separate days at doses of 25, 50, and 100 mg/kg/day body weight did not result in mortality, nor were there any study drug-related effects on clinical observations (**Table S11**). Incidences of hair loss, scabs/crust, abnormal skin color, abrasions and bruising were observed in various groups, including vehicle control animals. Additional observations of soft/liquid feces and red discharge (nostril, anus, urogenital area) were limited in duration, sporadic in nature and presented in various groups, including vehicle groups or in acclimation. A relationship to Adrecizumab was thought to be unlikely due to the sporadic nature of the observations, presence in the vehicle group and/or prior to treatment initiation, the limited number of animals involved and the lack of a dose-response relationship.

Body weight and food intake

There appeared to be no Adrecizumab-related effects on body weight (**Figure 7**) or food intake (data not shown; food intake was normal in all animals on every study day).

Neurological observations

There were no Adrecizumab-related effects on the neurological tests performed (**Table S12**). Neurological observations were normal, except for one female in the 25 mg/kg/day group who presented with head tilt before treatment administration and on day 15.

Electrocardiography

There were no Adrecizumab-related effects on ECG parameters. All ECGs were quantitatively within normal limits (**Table S13**) and not affected by Adrecizumab. One female animal from the 50 mg/kg/day group showed a right bundle branch block pattern before treatment administration and on day 14 (which is a normal variant in primates). One animal from the 100 mg/kg/day had 10 ventricular premature complexes on day 14 (a sporadic finding that is likely not related to Adrecizumab; this is observed in low incidence in cynomolgus monkeys). No other arrhythmias were observed. No Adrecizumab-related effects on the RR, PR, QRS, QT intervals, or QTc were found at any dose levels based on comparison of predose and postdose group values (**Table S13**).

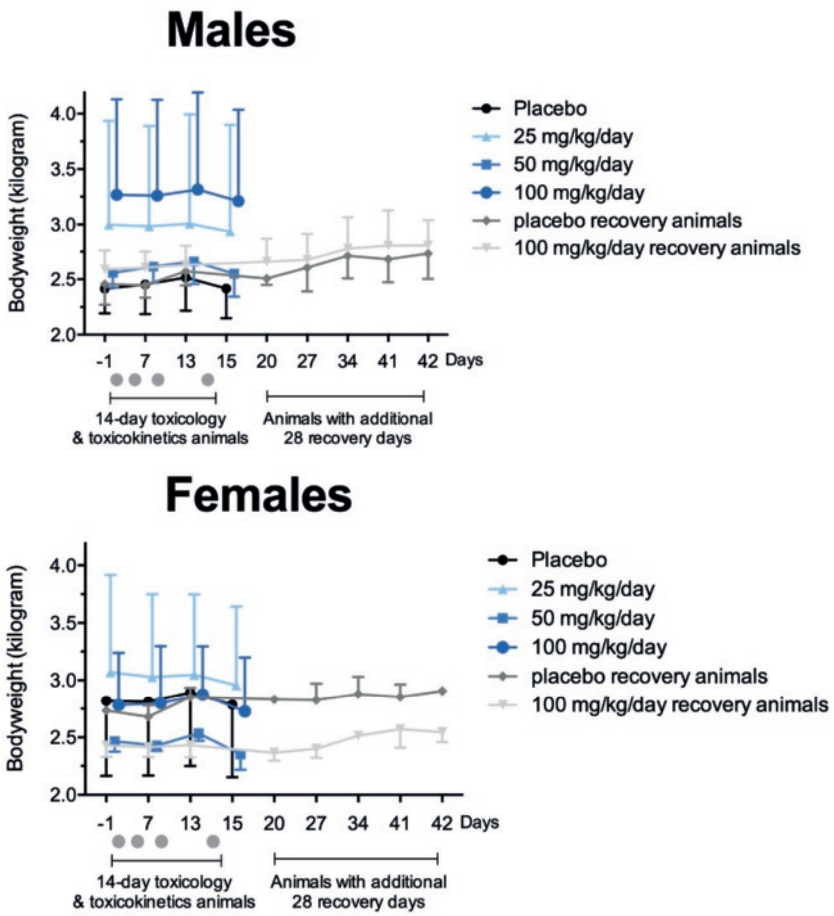


Figure 7. Bodyweight in cynomolgus monkeys. Data are expressed as mean ± SD. Grey dots indicate days on which study drug was administered (day 1, 4, 8 and 14).

Blood pressure and respiratory rate

There were no Adrecizumab-related effects on systolic and diastolic blood pressure or on respiratory rate (**Table S14**).

Ophthalmology

There were no Adrecizumab-related effects noted in ophthalmology examinations. Three animals had lesions that were already present before treatment administration, which were unchanged on day 14 and therefore not considered Adrecizumab-related (1 male animal from the 25 mg/kg/day group: anterior and posterior punctuate cataract in both eyes, 1 female animal from the 25 mg/kg/day group: moderate retinal vessel tortuosity in both eyes, and 1 female animal from the 100 mg/kg/day group: mild temporal optic nerve atrophy in both eyes). Punctate posterior and/or anterior cortical cataracts are seen frequently in mature cynomolgus monkeys as is the spontaneous findings, of varying severity, of temporal optic nerve atrophy. The retinal blood vessel tortuosity observed in one animal appeared to be an anomalous development.

Clinical pathology

There appeared to be no Adrecizumab-related effects on urinalysis (**Table S15**), hematology (**Table 4**), serum chemistry and coagulation (**Table 5**), as well as blood gas parameters (**Table S16**). One female animal from the 50 mg/kg/day dose group exhibited low platelets (55 * 103/µL) and a low neutrophil count (0.18 * 103/ µL) on day 15, however, similar findings were not observed in any of the remaining animals, and therefore this was considered a coincidental finding, likely due to preanalytical factors.

Serum chemistry findings (**Table 5**) prior to study drug administration and on day 15 included increases of AST, ALT and CK, in animals from all groups, including the vehicle group. These increases were not observed on day 41 in recovery animals. The coordinated nature of these findings and predominant increase in CK and AST are suggestive of skeletal muscle injury common to restraint and sampling. Additional variations were inconsistently observed during acclimation and on day 14 in blood gas parameters, which were considered procedural and not Adrecizumab-related.

Lymphocyte phenotyping and cytokines

There appeared to be no Adrecizumab-related effects on flow cytometry

parameters (**Table S17A** and **B**). Intra-group variability was observed in all groups for T cells (including T helper, T cytotoxic, T regulatory), B cells, and NK cells (including the activated subset) over the course of the study and therefore no consistent change in relationship to Adrecizumab administration was observed for the parameters analyzed.

Cytokines

Treatment with 100 mg/kg/day Adrecizumab did appear to not affect plasma cytokine levels (Table S18). No relevant changes were observed in the levels of IL-2, IL-6, IL-8, IL-12 and IFNγ.

Antibody-formation (IgE)

There were no apparent Adrecizumab-related effects on serum levels of IgE (**Table S19**). Total IgE exhibited intra- and inter- group variability, and the highest total IgE levels were observed in individual vehicle group animals. There were no dose-related effects.

Pathology

There were no Adrecizumab-related effects on gross pathology (**Table S20**), organ weights (**Table S21**) and histopathological evaluation (**Table S22**).

Table 4. Hemotology in cynomolgus monkeys.

Day	Parameter	Male				Female			
		Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day	Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day
Day -6	No. of animals	n=5	n=3	n=3	n=5	n=5	n=3	n=3	n=5
	RBC (10^6/mL)	5.77 ± 0.28	5.26 ± 0.52	5.86 ± 0.09	5.55 ± 0.33	5.69 ± 0.48	5.64 ± 0.09	5.41 ± 0.28	5.48 ± 0.50
	Hb (g/dL)	12.9 ± 0.2	12.3 ± 1.1	13.2 ± 0.2	13.2 ± 0.6	13.2 ± 0.6	13.2 ± 0.8	12.9 ± 1.0	12.7 ± 0.6
	Ht (%)	43.7 ± 1.4	40.7 ± 2.7	44.2 ± 0.7	43.5 ± 2.4	45.5 ± 3.1	42.4 ± 2.2	42.1 ± 2.1	42.3 ± 2.0
	MCV (fL)	76.0 ± 2.7	77.7 ± 3.3	75.5 ± 1.3	78.4 ± 2.7	80.0 ± 1.8	75.2 ± 2.9	77.9 ± 2.9	77.6 ± 4.1
	MCH (pg)	22.5 ± 1.2	23.3 ± 0.5	22.6 ± 0.1	23.7 ± 1.4	23.3 ± 1.0	23.3 ± 1.0	23.8 ± 1.0	23.2 ± 1.2
	MCHC (g/dL)	29.6 ± 0.7	30.0 ± 0.7	29.9 ± 0.5	30.3 ± 0.9	29.2 ± 0.8	31.0 ± 0.6	30.6 ± 0.9	30.0 ± 0.6
	RBCdw (%)	13.0 ± 1.7	12.7 ± 1.0	12.5 ± 0.9	11.9 ± 0.7	12.3 ± 0.6	12.4 ± 0.4	11.8 ± 0.3	12.1 ± 0.4
	Reticulocytes (%)	1.2 ± 0.5	1.6 ± 0.6	1.5 ± 0.4	1.3 ± 0.3	1.1 ± 0.4	1.3 ± 0.4	1.1 ± 0.2	1.0 ± 4.0
	Reticulocytes (10^6/mL)	0.066 ± 0.029	0.085 ± 0.019	0.088 ± 0.019	0.070 ± 0.019	0.063 ± 0.018	0.074 ± 0.024	0.058 ± 0.008	0.053 ± 0.016
	PLT (10^3/mL)	417 ± 48	377 ± 54	354 ± 42	387 ± 121	427 ± 62	437 ± 117	362 ± 21	390 ± 116
	MPV (fL)	9.2 ± 0.3	9.7 ± 1.2	9.9 ± 0.4	9.7 ± 0.9	9.6 ± 1.0	8.7 ± 0.6	9.9 ± 0.4	9.2 ± 0.7
	WBC (10^3/mL)	15.37 ± 5.55	11.20 ± 1.63	8.76 ± 1.47	14.28 ± 6.78	11.31 ± 3.11	11.94 ± 3.46	10.54 ± 2.45	12.51 ± 3.55
	Neutrophils (10^3/mL)	8.72 ± 6.16	7.18 ± 2.39	4.35 ± 0.64	8.55 ± 6.57	6.15 ± 1.35	7.71 ± 4.39	5.79 ± 2.97	7.80 ± 3.75
	Lymphocytes (10^3/mL)	6.04 ± 1.64	3.58 ± 1.46	4.00 ± 1.71	5.28 ± 3.16	4.70 ± 2.00	3.89 ± 1.45	4.34 ± 2.21	4.23 ± 1.52
	Monocytes (10^3/mL)	0.39 ± 0.09	0.34 ± 0.19	0.28 ± 0.08	0.31 ± 0.12	0.31 ± 0.08	0.23 ± 0.05	0.30 ± 0.14	0.30 ± 0.11
	Basophils (10^3/mL)	0.08 ± 0.02	0.04 ± 0.01	0.04 ± 0.03	0.06 ± 0.05	0.06 ± 0.02	0.06 ± 0.03	0.05 ± 0.03	0.06 ± 0.02
	Eosinophils (10^3/mL)	0.07 ± 0.05	0.03 ± 0.04	0.04 ± 0.03	0.03 ± 0.03	0.04 ± 0.06	0.01 ± 0.00	0.02 ± 0.01	0.08 ± 0.15

Table 4. continued.

Day	Parameter	Male				Female			
		Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day	Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day
Day 15	No. of animals	n=5	n=3	n=3	n=5	n=5	n=3	n=3	n=5
	RBC (10^6/mL)	5.26 ± 0.54	5.17 ± 0.42	5.31 ± 0.24	5.19 ± 0.38	5.11 ± 0.31	5.17 ± 0.37	5.30 ± 0.17	5.01 ± 0.30
	Hb (g/dL)	11.5 ± 1.0	11.7 ± 0.6	11.7 ± 0.6	12.2 ± 0.7	11.7 ± 0.4	11.9 ± 1.3	12.5 ± 0.4	11.6 ± 0.7
	Ht (%)	39.7 ± 3.0	40.3 ± 1.8	4.0 ± 1.2	40.6 ± 2.1	40.1 ± 1.9	38.6 ± 3.7	40.4 ± 0.3	38.8 ± 2.0
	MCV (fL)	75.6 ± 3.0	78.1 ± 2.9	75.3 ± 1.2	78.2 ± 3.0	78.6 ± 2.0	74.5 ± 2.0	76.4 ± 3.1	77.4 ± 3.8
	MCH (pg)	21.9 ± 1.2	22.8 ± 0.8	21.9 ± 0.3	23.5 ± 1.3	22.9 ± 1.0	23.1 ± 0.8	23.6 ± 1.4	23.1 ± 1.1
	MCHC (g/dL)	29.0 ± 0.5	29.2 ± 0.3	29.1± 0.6	30.1 ± 0.7	29.1 ± 0.7	31.0 ± 0.8	30.8 ± 0.7	29.9 ± 0.9
	RBCdw (%)	14.1 ± 1.9	12.7 ± 0.3	12.9 ± 0.8	12.9 ± 0.8	13.6 ± 0.7	13.2 ± 1.2	12.2 ± 0.5	13.0 ± 0.7
	Reticulocytes (%)	2.0 ± 0.7	1.6 ± 0.7	1.7 ± 0.6	1.9 ± 0.6	2.1 ± 0.4	1.9 ± 0.2	1.4 ± 0.2	1.7 ± 0.9
	Reticulocytes (10^6/mL)	0.102 ± 0.035	0.081 ± 0.038	0.090 ± 0.032	0.098 ± 0.030	0.108 ± 0.015	0.097 ± 0.013	0.077 ± 0.009	0.084 ± 0.041
	PLT (10^3/mL)	390 ± 49	360 ± 52	324 ± 49	370 ± 108	402 ± 55	444 ± 102	250 ± 176	363 ± 78
	MPV (fL)	9.1 ± 0.3	9.8 ± 0.7	9.6 ± 0.5	9.4 ± 0.9	9.0 ± 1.1	8.7 ± 0.4	11.0 ± 2.7	8.9 ± 0.6
	WBC (10^3/mL)	12.90 ± 2.34	12.36 ± 0.82	8.24 ± 1.43	12.56 ± 2.89	11.05 ± 1.01	11.55 ± 1.12	12.31 ± 2.52	12.31 ± 2.52
	Neutrophils (10^3/mL)	6.64 ± 2.02	7.41 ± 0.32	3.90 ± 0.08	7.20 ± 2.47	6.36 ± 1.42	7.26 ± 2.20	4.07 ± 3.37	7.88 ± 2.27
	Lymphocytes (10^3/mL)	5.63 ± 1.48	4.39 ± 0.35	3.96 ± 1.32	4.86 ± 1.17	4.20 ± 1.41	3.84 ± 1.17	3.48 ± 1.47	3.83 ± 1.55
	Monocytes (10^3/mL)	0.49 ± 0.17	0.34 ± 0.11	0.28 ± 0.03	0.38 ± 0.10	0.35 ± 0.11	0.36 ± 0.02	0.20 ± 0.14	0.40 ± 0.15
	Basophils (10^3/mL)	0.05 ± 0.02	0.05 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.05 ± 0.02	0.04 ± 0.01	0.04 ± 0.02	0.03 ± 0.02
	Eosinophils (10^3/mL)	0.04 ± 0.06	0.12 ± 0.11	0.03 ± 0.03	0.03 ± 0.02	0.06 ± 0.06	0.01 ± 0.01	0.03 ± 0.03	0.11 ± 0.18

Table 4. continued.

Day	Parameter	Male				Female			
		Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day	Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day
Day 41 (recovery animals)	No. of animals	n=2	n=0	n=0	n=2	n=2	n=0	n=0	n=2
	RBC (10^6/mL)	5.70 ± 0.27			6.31 ± 0.13	5.27 ± 0.50			5.86 ± 0.57
	Hb (g/dL)	12.6 ± 0.4			14.4 ± 0.6	12.4 ± 1.3			13.0 ± 0.7
	Ht (%)	42.8 ± 1.1			48.2 ± 2.6	41.6 ± 4.5			42.5 ± 4.0
	MCV (fL)	75.1 ± 1.6			76.4 ± 5.6	78.9 ± 0.9			72.5 ± 0.1
	MCH (pg)	22.2 ± 0.4			22.8 ± 1.3	23.6 ± 0.3			22.3 ± 0.9
	MCHC (g/dL)	29.5 ± 0.1			30.0 ± 0.5	29.9 ± 0.0			30.7 ± 1.2
	RBCdw (%)	14.2 ± 1.9			12.3 ± 0.6	12.2 ± 0.5			12.0 ± 0.0
	Reticulocytes (%)	1.1 ± 0.1			1.1 ± 0.3	0.9 ± 0.0			1.0 ± 0.2
	Reticulocytes (10^6/mL)	0.060 ± 0.008			0.069 ± 0.013	0.048 ± 0.007			0.054 ± 0.008
	PLT (10^3/mL)	466 ± 78			382 ± 48	423 ± 69			380 ± 112
	MPV (fL)	9.4 ± 0.5			9.0 ± 0.6	8.6 ± 0.0			9.5 ± 0.1
	WBC (10^3/mL)	15.64 ± 0.85			14.89 ± 4.99	17.23 ± 8.68			13.16 ± 4.89
	Neutrophils (10^3/mL)	4.67 ± 1.12			3.88 ± 0.61	6.93 ± 4.16			5.67 ± 0.65
	Lymphocytes (10^3/mL)	9.48 ± 1.44			9.81 ± 5.74	8.98 ± 3.42			6.81 ± 4.09
	Monocytes (10^3/mL)	0.79 ± 0.09			0.50 ± 0.09	0.55 ± 0.33			0.47 ± 0.06
	Basophils (10^3/mL)	0.12 ± 0.01			0.12 ± 0.08	0.14 ± 0.08			0.06 ± 0.04
	Eosinophils (10^3/mL)	0.51 ± 0.43			0.49 ± 0.20	0.54 ± 0.65			0.07 ± 0.01

Data are expressed as mean ± SD.

Abbreviations: hematocrit (Ht), hemoglobin (Hb), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean platelet volume (MPV), platelets, red blood cells (RBC), red cell distribution width (RBCW), reticulocytes absolute & percentual, white blood cells (WBC) and differential leukocyte count (absolute). No statistical analysis was performed because of low group sizes.

Table 5. Clinical chemistry and coagulation in cynomolgus monkeys.

Day	Parameter	Male				Female			
		Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day	Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day
Day -6	No. of animals	n=5	n=3	n=3	n=5	n=5	n=3	n=3	n=5
	Total protein (g/dL)	6.9 ± 0.4	6.3 ± 0.5	7.0 ± 0.4	6.9 ± 0.5	7.2 ± 0.4	7.0 ± 0.2	6.7 ± 0.4	7.0 ± 0.3
	ALB (g/dL)	4.1 ± 0.1	4.1 ± 0.4	4.4 ± 0.1	4.2 ± 0.5	4.3 ± 0.2	4.2 ± 0.2	4.1 ± 0.2	4.1 ± 0.1
	Globulin (g/dL)	2.9 ± 0.3	2.2 ± 0.1	2.6 ± 0.3	2.6 ± 0.4	2.9 ± 0.4	2.8 ± 0.4	2.6 ± 0.6	2.9 ± 0.3
	A/G	1.4 ± 0.2	1.9 ± 0.1	1.7 ± 0.2	1.6 ± 0.2	1.5 ± 0.2	1.5 ± 0.3	1.6 ± 0.4	1.4 ± 0.1
	ALT (U/L)	45 ± 8	89 ± 78	45 ± 12	43 ± 10	50 ± 30	48 ± 16	34 ± 15	39 ± 11
	AST (U/L)	48 ± 14	90 ± 105	61 ± 38	31 ± 4	33 ± 10	40 ± 10	32 ± 7	37 ± 14
	CK (U/L)	187 ± 77	672 ± 868	372 ± 332	161 ± 51	176 ± 62	182 ± 61	135 ± 37	208 ± 157
	ALP (U/L)	544 ± 150	462 ± 93	574 ± 162	512 ± 168	388 ± 62	322 ± 160	439 ± 153	393 ± 110
	GGT (U/L)	81 ± 13	73 ± 16	79 ± 25	74 ± 15	55 ± 18	50 ± 3	65 ± 11	52 ± 11
	TBIL (mg/dL)	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
	Gluc (mg/dL)	65 ± 22	56 ± 28	80 ± 8	54 ± 16	66 ± 16	64 ± 18	61 ± 5	62 ± 8
	TChol (mg/dL)	125 ± 20	101 ± 12	133 ± 23	125 ± 41	131 ± 36	119 ± 6	122 ± 36	122 ± 13
	TGC (mg/dL)	47 ± 4	39 ± 6	36 ± 1	42 ± 14	47 ± 10	57 ± 23	41 ± 15	39 ± 10
	Urea nitrogen (mg/dL)	22 ± 2	20 ± 8	21 ± 3	21 ± 3	19 ± 4	19 ± 9	24 ± 3	21 ± 3
	CRN (mg/dL)	0.7 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.0	0.7 ± 0.2	0.7 ± 0.1	0.6 ± 0.0
	CA (mg/dL)	9.6 ± 0.4	9.4 ± 0.5	9.6 ± 0.2	9.8 ± 0.3	10.3 ± 0.3	10.0 ± 0.4	9.7 ± 0.3	9.8 ± 0.2
	Inorganic phosphorus (mg/dL)	5.8 ± 0.7	5.1 ± 0.3	6.0 ± 0.7	5.9 ± 0.8	5.5 ± 0.6	4.4 ± 0.9	5.6 ± 0.7	5.3 ± 0.3
	Sodium (mEq/L)	145 ± 2	145 ± 3	144 ± 3	145 ± 2	142 ± 3	144 ± 1	144 ± 3	145 ± 1
	Potassium (mEq/L)	4.7 ± 0.7	4.5 ± 0.3	5.7 ± 2.0	4.3 ± 0.6	4.8 ± 0.4	4.5 ± 0.2	4.1 ± 0.2	4.3 ± 0.5
	Chloride (mEq/L)	109 ± 2	110 ± 1	107 ± 1	106 ± 1	109 ± 2	107 ± 3	106 ± 2	108 ± 2
	IgG (mg/dL)	991 ± 171	762 ± 14	893 ± 72	820 ± 100	1008 ± 161	831 ± 75	875 ± 349	995 ± 158
	PT (s)	11.3 ± 0.3	11.3 ± 0.4	11.1 ± 0.7	11.2 ± 0.7	11.3 ± 0.4	10.5 ± 0.6	10.7 ± 0.6	10.9 ± 0.2
	APTT (s)	19.4 ± 1.2	17.3 ± 1.3	17.0 ± 1.0	18.7 ± 2.2	20.5 ± 1.5	19.6 ± 3.4	17.7 ± 0.7	19.1 ± 0.7
	Fibrinogen (mg/dL)	268 ± 64	235 ± 15	209 ± 8	247 ± 48	234 ± 16	254 ± 74	226 ± 29	220 ± 22

Table 5. continued.

Day	Parameter	Male				Female			
		Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day	Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day
Day 15	No. of animals	n=5	n=3	n=3	n=5	n=5	n=3	n=3	n=5
	Total protein (g/dL)	7.0 ± 0.3	6.4 ± 0.4	6.7 ± 0.4	7.0 ± 0.4	6.9 ± 0.3	6.7 ± 0.4	6.8 ± 0.2	6.8 ± 0.3
	ALB (g/dL)	4.3 ± 0.2	4.2 ± 0.5	4.3 ± 0.1	4.3 ± 0.2	4.3 ± 0.1	4.2 ± 0.3	3.9 ± 0.3	4.2 ± 0.1
	Globulin (g/dL)	2.7 ± 0.3	2.2 ± 0.1	2.4 ± 0.3	2.7 ± 0.3	2.6 ± 0.3	2.5 ± 0.3	2.9 ± 0.2	2.6 ± 0.3
	A/G	1.6 ± 0.3	1.9 ± 0.2	1.8 ± 0.3	1.7 ± 0.2	1.7 ± 0.2	1.7 ± 0.3	1.4 ± 0.2	1.6 ± 0.2
	ALT (U/L)	72 ± 35	88 ± 58	80 ± 12	81 ± 14	104 ± 58	91 ± 47	53 ± 26	57 ± 17
	AST (U/L)	173 ± 117	165 ± 138	161 ± 23	164 ± 69	161 ± 120	170 ± 139	122 ± 16	118 ± 39
	CK (U/L)	6445 ± 7730	3363 ± 2432	8346 ± 10394	4802 ± 3050	3751 ± 3960	3939 ± 5629	2245 ± 1143	3591 ± 2035
	ALP (U/L)	496 ± 129	416 ± 160	501 ± 145	424 ± 100	362 ± 60	345 ± 131	419 ± 161	357 ± 106
	GGT (U/L)	72 ± 10	52 ± 10	65 ± 24	59 ± 16	45 ± 16	46 ± 2	53 ± 5	46 ± 9
	TBIL (mg/dL)	0.3 ± 0.1	0.3 ± 0.0	0.5 ± 0.2	0.5 ± 0.4	0.4 ± 0.2	0.5 ± 0.1	0.4 ± 0.2	0.5 ± 0.3
	Gluc (mg/dL)	47 ± 13	49 ± 2	51 ± 16	42 ± 12	50 ± 6	39 ± 9	48 ± 9	43 ± 8
	Tchol (mg/dL)	122 ± 13	91 ± 7	103 ± 8	108 ± 18	124 ± 34	105 ± 7	99 ± 21	110 ± 16
	TGC (mg/dL)	41 ± 11	40 ± 1	42 ± 18	44 ± 14	44 ± 11	51 ± 5	38 ± 9	40 ± 8
	Urea nitrogen (mg/dL)	24 ± 6	23 ± 6	23 ± 7	24 ± 6	21 ± 6	19 ± 3	27 ± 4	22 ± 3
	CRN (mg/dL)	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.2	0.6 ± 0.0	0.7 ± 0.2	0.7 ± 0.1	0.6 ± 0.1
	CA (mg/dL)	9.5 ± 0.4	9.3 ± 0.4	9.3 ± 0.2	9.5 ± 0.4	9.8 ± 0.2	9.7 ± 0.3	9.5 ± 0.3	9.6 ± 0.2
	Inorganic phosphorus (mg/dL)	9.6 ± 0.9	6.5 ± 0.8	6.6 ± 0.7	6.1 ± 0.8	5.7 ± 1.0	5.5 ± 0.3	4.4 ± 1.5	5.6 ± 0.6
	Sodium (mEq/L)	145 ± 1	147 ± 2	147 ± 2	145 ± 2	142 ± 3	144 ± 1	144 ± 3	145 ± 1
	Potassium (mEq/L)	4.5 ± 0.5	4.6 ± 0.6	4.7 ± 0.4	4.3 ± 0.3	4.3 ± 0.4	4.4 ± 0.2	4.0 ± 0.3	4.3 ± 0.3
	Chloride (mEq/L)	110 ± 1	110 ± 2	109 ± 1	109 ± 2	108 ± 3	109 ± 2	108 ± 6	110 ± 2
	IgG (mg/dL)	993 ± 188	815 ± 22	932 ± 72	1022 ± 155	960 ± 140	821 ± 73	1020 ± 181	1051 ± 144
	PT (s)	11.7 ± 0.3	11.9 ± 0.4	12.2 ± 0.5	11.6 ± 0.6	11.3 ± 0.3	10.8 ± 0.5	10.6 ± 0.5	11.2 ± 0.3
	APTT (s)	19.2 ± 1.5	18.4 ± 0.9	17.6 ± 1.5	18.2 ± 1.1	19.8 ± 2.8	19.5 ± 2.9	19.0 ± 2.5	18.4 ± 1.4
	Fibrinogen (mg/dL)	238 ± 38	224 ± 30	249 ± 22	232 ± 28	246 ± 31	274 ± 83	325 ± 150	221 ± 24

Table 5. continued.

Day	Parameter	Male				Female			
		Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day	Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day
Day 41 (recovery animals)	No. of animals	n=2	n=0	n=0	n=2	n=2	n=0	n=0	n=2
	Total protein (g/dL)	7.0 ± 0.6			7.3 ± 0.9	7.0 ± 0.4			7.0 ± 0.1
	ALB (g/dL)	3.7 ± 0.1			4.3 ± 0.3	3.8 ± 0.1			4.1 ± 0.1
	Globulin (g/dL)	3.3 ± 0.7			3.0 ± 0.6	3.2 ± 0.5			3.0 ± 0.2
	A/G	1.1 ± 0.3			1.5 ± 0.2	1.3 ± 0.2			1.4 ± 0.1
	ALT (U/L)	43 ± 11			30 ± 11	31 ± 1			33 ± 8
	AST (U/L)	46 ± 13			28 ± 3	23 ± 1			29 ± 9
	CK (U/L)	165 ± 52			213 ± 95	140 ± 5			114 ± 9
	ALP (U/L)	525 ± 95			484 ± 223	283 ± 37			289 ± 58
	GGT (U/L)	86 ± 6			73 ± 5	47 ± 10			51 ± 1
	TBIL (mg/dL)	0.2 ± 0.0			0.3 ± 0.1	0.2 ± 0.1			0.2 ± 0.1
	Gluc (mg/dL)	56 ± 20			73 ± 17	53 ± 2			48 ± 13
	Tchol (mg/dL)	113 ± 28			153 ± 33	143 ± 41			131 ± 21
	TGC (mg/dL)	48 ± 3			41 ± 13	56 ± 3			58 ± 13
	Urea nitrogen (mg/dL)	18 ± 0			21 ± 1	19 ± 3			22 ± 5
	CRN (mg/dL)	0.7 ± 0.1			0.8 ± 0.1	0.7 ± 0.1			0.7 ± 0.1
	CA (mg/dL)	9.3 ± 0.2			10.1 ± 0.1	9.6 ± 0.2			10.2 ± 0.4
	Inorganic phosphorus (mg/dL)	6.1 ± 0.3			6.8 ± 0.1	5.7 ± 0.9			4.7 ± 2.1
	Sodium (mEq/L)	143 ± 1			145 ± 3	142 ± 1			141 ± 0
	Potassium (mEq/L)	4.7 ± 0.3			4.5 ± 0.5	4.0 ± 0.0			3.9 ± 0.3
	Chloride (mEq/L)	108 ± 1			105 ± 0	106 ± 2			107 ± 4
	IgG (mg/dL)	1154 ± 390			1081 ± 380	1034 ± 100			910 ± 57
	PT (s)	11.8 ± 0.4			11.0 ± 0.6	10.8 ± 0.8			10.8 ± 0.2
	APTT (s)	22.3 ± 1.2			21.7 ± 1.7	20.8 ± 1.6			19.0 ± 0.6
	Fibrinogen (mg/dL)	214 ± 25			195 ± 8	287 ± 8			195 ± 22

Data are expressed as mean ± SD.

Abbreviations: Activated partial thromboplastin time (APTT), alanine aminotransferase (ALT), albumin (ALB) albumin/globulin ratio (A/G), alkaline phosphatase (ALP), aspartate aminotransferase (AST), calcium (CA), creatine kinase (CK), creatinine (CRN), gamma glutamyltransferase (GGT), immunoglobulin G (IgG), prothrombin time (PT), (APTT), total bilirubin (TBIL), total cholesterol (TChol), triglyceride (TGC). No statistical analysis was performed because of low group sizes.

Toxicokinetics

Plasma Adrecizumab concentration time-profiles of male and female monkeys are presented in **Figure 8**, and toxicokinetic parameters are depicted in **Table S23**. None of the samples collected from vehicle treated animals contained detectable Adrecizumab, indicating that there was no cross-contamination between the vehicle and Adrecizumab-treated groups (data not shown). Plasma kinetics of Adrecizumab was not influenced by gender. In all three dose groups, the Cmax was attained virtually immediately after termination of infusion. The dose-normalized maximum concentrations (Cmax/dose) and dose-normalized AUC0-24h (AUC0-24h/dose) showed dose-proportionality on days 1 and 14. The resulting rate of accumulation (Racc) was approximately 2. Adrecizumab was slowly eliminated from the circulation. The apparent elimination half-lives (T1/2) were calculated in a follow-up period of 72 hours after the first dose administration, and in the period of 24 hours of follow-up after the final dose administration. This resulted in T1/2 of approximately 80 and 40 hours, respectively. However, because there appeared to be a more rapid elimination phase in the first 6 hours post dose administration, and the follow-up period for the final dose administration was only 24 hours, the latter half-life may be less reliable. Due to uncertainty of the half-life determination, the weak fitting for a part of the curves and extremely high extrapolations to infinity, the AUC0-inf should not be viewed as a reliable parameter.

Circulating adrenomedullin concentrations

Administration of Adrecizumab resulted in a rapid and dose-dependent increase of circulating levels of total ADM (the assay detects both free and Adrecizumab-bound ADM; **Supplementary Figure 3**).

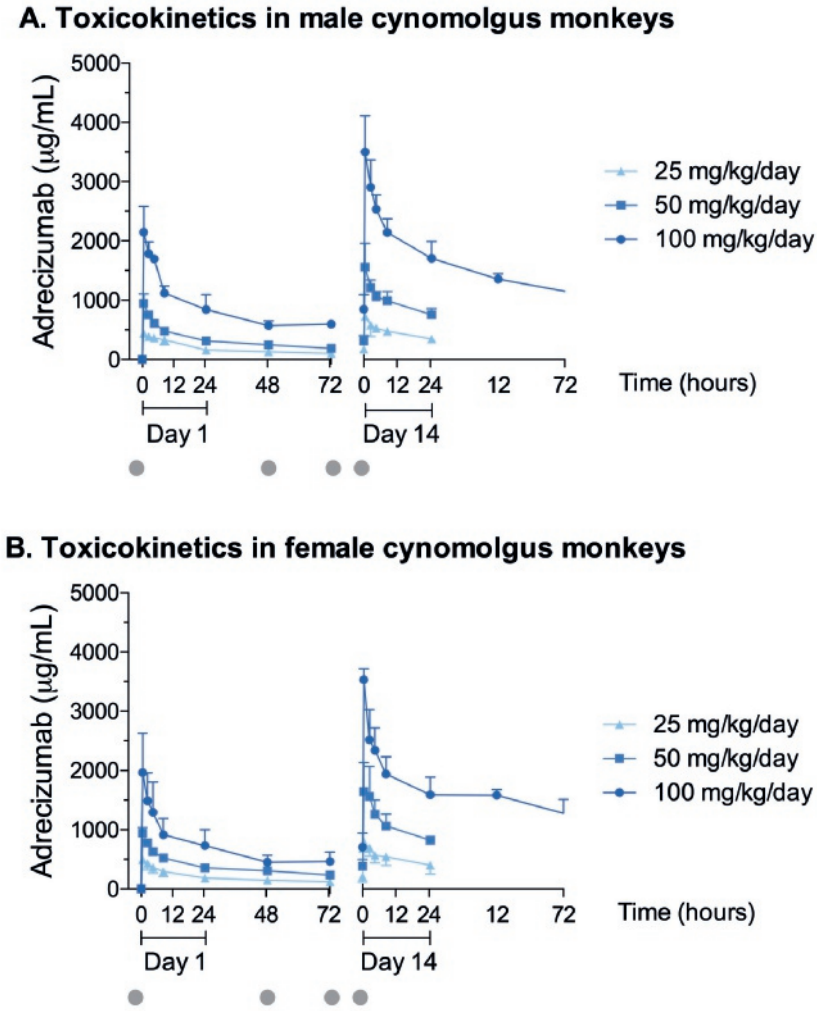


Figure 8. Toxicokinetics in cynomolgus monkeys. Grey dots indicate days on which study drug was administered (day 1, 4, 8 and 14). Data are expressed as mean ± SD.

Discussion

In the present study, we report the toxicological profile and toxicokinetics of Adrecizumab in rodents, dogs and non-human primates during and after repeated-dose administration over a period of 14 days. Adrecizumab was well tolerated in doses of up to 400 mg/kg/day in Wistar rats, 50 mg/kg in beagle dogs and 100 mg/kg/day in cynomolgus monkeys (note that these no-observed-adverse-effect-levels [NOAEL] are 200, 25 and 50 times greater than the intended therapeutic dose of 2 mg/kg in humans). No toxicologically significant alterations could be attributed to Adrecizumab. Toxicokinetic analysis revealed that peak concentrations were attained almost immediately upon cessation of Adrecizumab administration, that peak concentrations and area-under-curve increased in a dose-proportional manner, and that accumulation ratios of Adrecizumab were fairly consistent.

Toxicology

Previously published efficacy experiments with a murine variant of the Adrecizumab antibody in mice did not show any safety concerns, although in this study only single doses were applied[18]. In the present work, no mortality or moribund conditions were observed in any of the animals. Furthermore, no Adrecizumab-related changes in any of the safety parameters (including vital signs, electrocardiography, ophthalmology, urinalysis, extensive blood analysis and [histo]pathological evaluation) were thought to be toxicologically significant due to lack of any clear indications of clinical adversity and the absence of dose-response relationships. Adrecizumab neither induced blood pressure alterations, nor arrhythmias. In the rat study, transient red discoloration of urine was observed in all vehicle-treated rats as well as in 6 animals treated with 100 mg/kg Adrecizumab, but not in 200 and 400 mg/kg groups. It appears most likely that transient hemolysis was responsible for the red discoloration of the urine, based on hypo-osmotic effects of the water for injection used to dissolve L-histidine (vehicle) powder, because this practically constitutes distilled water (i.e. with no electrolytes). Vehicle-treated animals only received this L-histidine/water for injection solution, while the 100 and 200 mg/kg groups received a mixture of the L-histidine/water for injection solution and Adrecizumab solution (in a 3:1 and 1:1 ratio, respectively). The 400 mg/kg group only received Adrecizumab solution. Since Adrecizumab was dissolved in PBS, and therefore less hypo-osmotic, this may have counterbalanced the hypo-osmotic effects of the water for injection. This may explain why the red discoloration of urine was observed

in all vehicle-treated groups (often classified as 'mild'), while only in a few animals of the 100 mg/kg groups (classified as 'slightly') and in none of the animals of the 200 or 400 mg/kg groups.

Toxicokinetics

Adrecizumab was administered intravenously on 4 separate occasions over a 14 day period. Toxicokinetics were analyzed after the first and last infusions. Importantly, no gender differences were observed. Peak concentrations were observed almost immediately after completion of infusion and increased in a dose-proportional manner. Adrecizumab was eliminated slowly from the circulation, which is typical for monoclonal antibodies[22,23]. Repeated administration of Adrecizumab resulted in quantifiable plasma levels before administration on day 14, and also increased total exposure on day 14. It was not possible to investigate half-life in rats because the duration of sample collection was limited (samples were only taken during the first 24 hours after the first and last Adrecizumab infusion). However, in cynomolgus monkeys, samples were taken at a few additional time-points. There appeared to be multi-phase elimination, with more rapid elimination over the first 6 hours. The elimination half-life in cynomolgus monkeys was 40 hours at 24 hours and 80 hours in the second phase at 24-72 hours, respectively.

Effects on the target peptide adrenomedullin

Interestingly, administration of Adrecizumab resulted in a swift, profound, and sustained increase of circulating ADM levels, which is in line with previous work and already shown not be the result of increased synthesis of the ADM peptide[20,21]. A mechanistic explanation for this effect was recently put forward[24]. In short, it is hypothesized that excess antibody in the circulation drains extravascular ADM from the interstitium into the circulation. This relies on the fact that ADM is small enough to easily cross the endothelial barrier, whereas the antibody is not, effectively "trapping" ADM in the circulation. Because Adrecizumab only marginally inhibits ADM signaling, the profoundly increased levels of ADM in the circulation (bound to Adrecizumab) are thought to result in an overall, "net", increase of ADM signaling in endothelial cells, thereby enhancing endothelial barrier function and reducing tissue edema. Furthermore, it is well known that high concentrations of ADM can cause hypotension through effects on vascular smooth muscle cells located on the interstitial site of arteries[13-15]. Hence, decreased concentrations of ADM in the interstitium may negate its

vasodilatory effects. In this context it is important to emphasize that, despite the fact that Adrecizumab administration resulted in very high circulating ADM levels in the present study, no effects on blood pressure parameters were observed in beagle dogs or cynomolgus monkeys. In human studies[21], administration of the intended therapeutic dose of Adrecizumab (a single dose of 2 mg/kg, a dose for which beneficial effects were observed in both published[18-20] and unpublished preclinical studies) resulted in immediate increases of circulating ADM concentrations of approximately 10- to 30-fold (during non-inflammatory circumstances and during human endotoxin-induced inflammation, respectively). This is similar to what was observed for animals that were administered 2 mg/kg[20], suggesting that this dose may also confer beneficial effects in humans.

A strength of the present work is the use of two different animal species and repeated administration of high doses of Adrecizumab (significantly higher than the intended single dose administration of approximately 0.5 to 8 mg/kg in humans). In addition, because the ADM peptide and receptors are highly conserved across species, the animals used in the present work are well-suited for toxicological evaluation of Adrecizumab. However, several limitations also need to be addressed. Although repeated administration of very high doses of Adrecizumab represent an excellent method to investigate safety, it may limit the translatability of toxicokinetic results to pharmacokinetics of single dose administration in humans.

Conclusion

Intravenous, repeated administration of high doses of Adrecizumab was safe and well-tolerated in rodents, dogs and non-human primates. In addition, Adrecizumab administration resulted in a swift and profound increase of circulating concentrations of its target peptide ADM, which is consistent with previous preclinical studies and may underpin its purported beneficial effects on endothelial barrier, as well as smooth muscle cell dysfunction during sepsis. These results pave the way for further investigation of Adrecizumab in humans.

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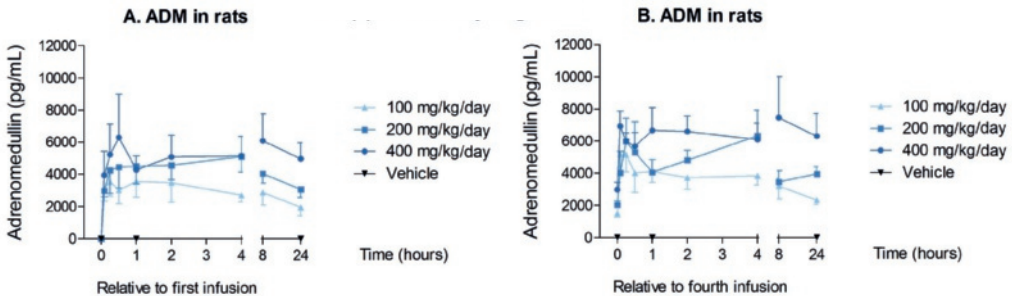
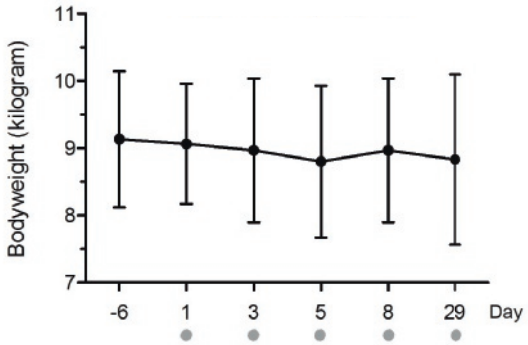
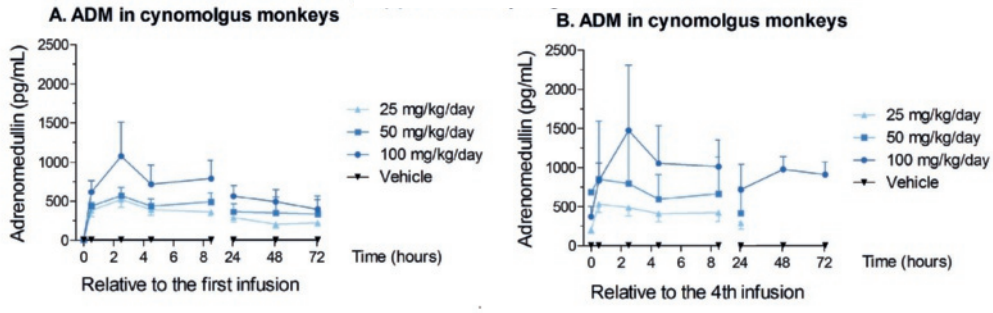


Supplementary Figure 1. Circulating total adrenomedullin concentrations in rats after the first (**A**) and fourth infusion (**B**). Data are expressed as mean ± SD.



Supplementary Figure 2. Body weight in Beagle dogs (n=3). Data are expressed as mean ± SD. Grey dots indicate days on which study drug was administered (day 1, 3, 5, 8 and 29).



Supplementary Figure 3. Circulating total adrenomedullin concentrations in Cynomolgus monkeys after the first (**A**) and fourth infusion (**B**). Data are expressed as mean ± SD.

Table S1. Clinical signs in early toxicology animals (rats).

Sex	Dose group (mg/kg/day)	No. of animals	Clinical sign in no. of animals	Study day													
				1	2	3	4	5	6	7	8	9	10	11	12	13	14
Male	0	15[A]	NOA	0	15	15	0	15	15	15	0	15	15	15	15	15	0
			Slight red discoloration of urine	14	0	0	15	0	0	0	15	0	0	0	0	0	15
			Moderate red discoloration of urine	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	100	10	NOA	8	10	10	10	10	10	10	10	10	10	10	10	10	10
			Slight red discoloration of urine	2	0	0	0	0	0	0	0	0	0	0	0	0	0
	200	10	NOA	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	400	15[A]	NOA	15	15	15	15	15	15	15	15	15	15	15	15	15	15
Female	0	15[A]	NOA	0	15	15	0	15	15	15	0	15	15	15	15	15	0
			Slight red discoloration of urine	0	0	0	0	0	0	0	15	0	0	0	0	0	15
			Moderate red discoloration of urine	15	0	0	15	0	0	0	0	0	0	0	0	0	0
	100	10	NOA	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	200	10	NOA	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	400	15[A]	NOA	15	15	15	15	15	15	15	15	15	15	15	15	15	15

[A] Includes the first 14 study days of the 5 recovery animals in the vehicle and 400 mg/kg group. *Abbreviations: NOA (no observable abnormality); no (number).*

Table S1B. Clinical signs in recovery animals (rats).

Sex	Dose group (mg/kg/day)	No. of animals	Clinical sign in no. of animals	Recovery day													
				1	2	3	4	5	6	7	8	9	10	11	12	13	14
Male	0	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	400	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Female	0	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	400	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Sex	Dose group (mg/kg/day)	No. of animals	Clinical sign in no. of animals	Recovery day													
				15	16	17	18	19	20	21	22	23	24	25	26	27	28
Male	0	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	400	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Female	0	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	400	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Abbreviations: NOA (no observable abnormality); no (number).

Table S1C. Clinical signs in toxicokinetics sattelite animals (rats).

Sex	Dose group (mg/kg/day)	No. of animals	Clinical sign in no. of animals	Study day													
				1	2	3	4	5	6	7	8	9	10	11	12	13	14
Male	0	3	NOA	0	3	3	0	3	3	3	0	3	3	3	3	3	0
			Slight red discoloration of urine	3	0	0	3	0	0	0	0	0	0	0	0	0	0
			Moderate red discoloration of urine	0	0	0	0	0	0	0	3	0	0	0	0	0	3
	100	9	NOA	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	200	9	NOA	9	9	9	9	9	9	9	8	8	8	8	8	8	8
			Multiple signs[B]	0	0	0	0	0	0	0	1	1	1	1	1	1	1
	400	9	NOA	9	9	9	9	9	9	9	9	9	9	9	9	9	9
Female	0	3	NOA	0	3	3	0	3	3	3	0	3	3	3	3	3	0
			Moderate red discoloration of urine	3	0	0	3	0	0	0	3	0	0	0	0	0	3
	100	9	NOA	5	9	9	9	9	9	9	9	9	9	9	9	9	9
			Slight red discoloration of urine	4	0	0	0	0	0	0	0	0	0	0	0	0	0
	200	9	NOA	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	400	9	NOA	9	9	9	9	9	9	9	9	9	9	9	9	9	9

[B] Decreased activity and body weight, porphyrin around nose and eyes, piloerection, pale mucosa and skin. *Abbreviations: NOA (no observable abnormality); no (number).*

Table S2. Summary of ophthalmological examinations in rats.

Sex	Dose group (mg/kg/day)	No. of animals	Examination	Before treatment	Treatment period	Additional recovery period
					Day 14	Day 42
Male	0	10	Normal	n=10	n=10	-
		5	Normal	n=5	n=5	n=5
	100	10	Normal	n=10	n=10	-
	200	10	Normal	n=10	n=10	-
	400	10	Normal	n=10	n=8	-
			Not possible[A]	n=0	n=1	-
			Not possible[B]	n=0	n=1	-
		5	Normal	n=5	n=5	n=5
Female	0	10	Normal	n=10	n=9	-
			Not possible[C]	n=0	n=1	-
		5	Normal	n=5	n=5	n=5
	100	10	Normal	n=10	n=10	-
	200	10	Normal	n=10	n=10	-
	400	10	Normal	n=10	n=10	-
		5	Normal	n=5	n=5	n=5

[A] Examination of right eye not possible in 1 animal, damage to left eye due to sampling.
[B] Examination of left eye not possible in 1 animal, damage to right eye due to sampling.
[C] Examination not possible in 1 animal, damage to both eyes due to sampling.
Abbreviations: no (number).

Table S3A. Urinalysis in male rats.

Parameter	Day 15 (n=10 per group)				42-day follow-up (n=5 per group)	
	Vehicle	100 mg/kg/day Adrecizumab	200 mg/kg/day Adrecizumab	400 mg/kg/day Adrecizumab	Vehicle	400 mg/kg/day Adrecizumab
Urine volume	42.2 ± 15.44	25.3 ± 12.89*	20.8 ± 9.07*	25.4 ± 14.05*	34.6 ± 10.09	21.2 ± 8.79
Specific gravity	1007 ± 4.73	1016 ± 10.41*	1019 ± 8.44*	1016 ± 5.68*	1018 ± 3.74	1021 ± 6.10
pH	7.1 ± 0.32	6.9 ± 0.74	7.0 ± 0.47	7.1 ± 0.26	7.0 ± 0.45	7.0 ± 0.00
RBC (microscopically)	0.7 ± 1.20	1.5 ± 2.55	4.2 ± 8.08	1.1 ± 1.85	0.4 ± 0.89	1.0 ± 1.41
WBC (microscopically)	0.2 ± 0.63	0.9 ± 1.52	2.5 ± 2.46	1.1 ± 1.71	1.4 ± 1.95	2.2 ± 1.48
Renal epithelial cells	0.1 ± 0.13	0.2 ± 0.63	0.6 ± 0.97	0.1 ± 0.52	0.4 ± 0.89	1.0 ± 1.41
Squamous epithelial cells	0.0 ± 0.00	0.9 ± 1.20	0.6 ± 0.97	0.5 ± 0.92	0 ± 0.00	0.8 ± 1.10
Casts	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Colour	Normal (n=10)	Normal (n=10)	Normal (n=10)	Normal (n=10)	Normal (n=5)	Normal (n=5)
Protein	- (n=10)	+ (n=1) - (n=9)	± (n=4) + (n=2) - (n=4)	± (n=6) + (n=3) - (n=1)	± (n=2) + (n=2) - (n=1)	+ (n=1) - (n=4)
BLD (Ery/µL)	+ (n=1) - (n=9)	+ (n=3)	+ (n=1) ++ (n=2) - (n=7)	+ (n=2) ++ (n=1) - (n=7)	+ (n=1) ++ (n=1) - (n=3)	- (n=5)
UBG (µmol/L)	Normal (n=10)	Normal (n=10)	Normal (n=10)	Normal (n=10)	Normal (n=5)	Normal (n=5)
BIL (µmol/L)	- (n=10)	- (n=10)	- (n=10)	- (n=10)	- (n=5)	- (n=5)
NIT (µmol/L)	+ (n=4) - (n=6)	+ (n=4) - (n=6)	+ (n=5) - (n=5)	+ (n=4) - (n=6)	+ (n=3) - (n=2)	+ (n=1) - (n=4)
KET (mmol/L)	- (n=10)	- (n=10)	- (n=10)	- (n=10)	- (n=5)	- (n=5)
GLU (mmol/L)	- (n=10)	- (n=10)	- (n=10)	- (n=10)	- (n=5)	- (n=5)
Leu (Leu/µL)	- (n=10)	+ (n=7) - (n=3)	+ (n=6) - (n=4)	+ (n=5) - (n=5)	+ (n=2) - (n=3)	+ (n=4) - (n=1)

Data are presented as mean ± SD. * $p \leq 0.05$ compared to vehicle.
Abbreviations: Bilirubin (BIL): + 17 µmol/L; ++ 35 µmol/L; +++ 70 µmol/L; blood (BLD): + 5-10 ery/µL; ++ 50 ery/µL; +++ 250 ery/µL; glucose (GLU): + 2.8 mmol/L; ++ 8.3 mmol/L; +++ >27.8 mmol/L; ketones (KIT): + 2.5 mmol/L; ++ 10 mmol/L; +++ 30 mmol/L; leucocytes (leu): + 25 leu/µL; ++ 75 leu/µL; +++ 500 leu/µL; nitrite (NIT): + positive (presence of nitrite-reducing microorganisms); n (number of animals); RBC (red blood cell); urobilinogen (UBG): + 35 µmol/L; ++ 70 µmol/L; +++ 140 µmol/L; ++++ 200 µmol/L; WBC (white blood cell).

Table S3B. Urinalysis in female rats.

Parameter	14 day follow-up (n=10 per group)				42-day follow-up (n=5 per group)	
	Vehicle	100 mg/kg/day Adrecizumab	200 mg/kg/day Adrecizumab	400 mg/kg/day Adrecizumab	Vehicle	400 mg/kg/day Adrecizumab
Urine volume	25.0 ± 13.00	22.9 ± 9.40	21.2 ± 6.71	17.2 ± 4.81	25.0 ± 14.4	18.8 ± 10.57
Specific gravity	1011 ± 4.99	1012.2 ± 50.03	1014.0 ± 4.99	1016.3 ± 3.28*	1018 ± 9.53	1020.4 ± 13.37
pH	7.0 ± 0.47	6.5 ± 0.43	6.5 ± 0.53	6.5 ± 0.52	7.0 ± 7.00	6.4 ± 0.55
RBC (microscopically)	1.0 ± 1.70	1.4 ± 1.58	0.6 ± 1.35	1.3 ± 1.49	0.4 ± 0.89	0.8 ± 1.10
WBC (microscopically)	0.5 ± 1.08	0.7 ± 1.16	1.5 ± 2.55	1.0 ± 1.73	1.2 ± 1.79	0.8 ± 1.10
Renal epithelial cells	0.6 ± 0.97	0.0 ± 0.00	0.2 ± 0.63	0.2 ± 0.70	0.4 ± 0.89	0.0 ± 0.00
Squamous epithelial cells	0.7 ± 1.00	1.0 ± 1.63	1.0 ± 1.41	0.3 ± 0.70	1.4 ± 1.34	0.8 ± 1.10
Casts	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Colour	Normal (n=10)	Normal (n=10)	Normal (n=10)	Normal (n=10)	Normal (n=5)	Normal (n=5)
Protein	- (n=10)	- (n=10)	- (n=10)	+ (n=1) - (n=10)	- (n=5)	+ (n=1) - (n=4)
BLD (Ery/µL)	+ (n=3) - (n=7)	+ (n=1) - (n=9)	- (n=10)	+ (n=3) ++ (n=1) - (n=6)	- (n=5)	++ (n=1) + (n=2) - (n=2)
UBG (µmol/L)	Normal (n=10)	Normal (n=10)	Normal (n=10)	Normal (n=10)	Normal (n=5)	Normal (n=5)
BIL (µmol/L)	- (n=10)	- (n=10)	- (n=10)	- (n=10)	- (n=5)	- (n=5)
NIT (µmol/L)	+ (n=8) - (n=2)	+ (n=9) - (n=1)	+ (n=5) - (n=5)	+ (n=3) - (n=7)	+ (n=2) - (n=3)	+ (n=1) - (n=4)
KET (mmol/L)	- (n=10)	- (n=10)	- (n=10)	- (n=10)	- (n=5)	- (n=5)
GLU (mmol/L)	- (n=10)	- (n=10)	- (n=10)	- (n=10)	- (n=5)	- (n=5)
Leu (Leu/µL)	- (n=10)	+ (n=1) - (n=9)	+ (n=1) - (n=9)	+ (n=2) - (n=8)	- (n=5)	- (n=5)

Data are presented as mean ± SD. * p ≤ 0.05 compared to vehicle.
Abbreviations: Bilirubin (BIL): + 17 µmol/L; ++ 35 µmol/L; +++ 70 µmol/L; blood (BLD): + 5-10 ery/µL; ++ 50 ery/µL; +++ 250 ery/µL; glucose (GLU): + 2.8 mmol/L; ++ 8.3 mmol/L; +++ >27.8 mmol/L; ketones (KIT): + 2.5 mmol/L; ++ 10 mmol/L; +++ 30 mmol/L; leucocytes (leu): + 25 leu/µL; ++ 75 leu/µL; +++ 500 leu/µL; nitrite (NIT): + positive (presence of nitrite-reducing microorganisms); n (number of animals); RBC (red blood cell); urobilinogen (UBG): + 35 µmol/L; ++ 70 µmol/L; +++ 140 µmol/L; ++++ 200 µmol/L; WBC (white blood cell).

Table S4. Lymphocyte phenotyping in rats.

Gender	Cell type		14 day follow-up (n=10 per group)							
			Vehicle (n=10)		100 mg/kg/day Adrecizumab (n=10)		200 mg/kg/day Adrecizumab (n=10)		400 mg/kg/day Adrecizumab (n=10)	
			Pre	Post	Pre	Post	Pre	Post	Pre	Post
Male	Total T-lymphocytes	$CD3^+$ (%)	40.77	43.25	41.82	41.13	43.99	39.66	45.01	40.78
	T-helper	$CD3^+$ $CD4^+$ (%)	28.67	29.23	29.00	27.75	29.26	25.53	30.42	26.76
	T-cytotoxic	$CD3^+$ $CD8a^+$ (%)	12.10	14.02	12.82	13.39	14.73	14.13	14.59	14.02
	NK T-cells	$CD3^+$ NKR-$P1A^+$ (%)	3.37	3.50	4.80*	3.75	4.06	2.66	4.29	3.63
	Activated T-cells	$CD3^-$ NKR-$P1A^+$ (%)	2.81	2.41	2.99	2.35	3.18	2.39	3.26	2.28
	NK cells	$CD3^-$ NKR-$P1A^+$ (%)	14.61	15.20	14.63	14.69	13.53	15.05	13.54	16.43
	B cells	$CD45RA^+$ cells (%)	37.41	29.73	35.75	30.99	35.85	33.08	38.16	36.91
Female	Total T-lymphocytes	$CD3^+$ (%)	45.65	47.45	47.54	44.77	51.01	47.56	46.08	43.48
	T-helper	$CD3^+$ $CD4^+$ (%)	32.72	35.35	35.18	32.99	37.01	34.33	34.65	32.05
	T-cytotoxic	$CD3^+$ $CD8a^+$ (%)	12.94	12.10	12.36	11.78	14.00	13.24	11.43	11.43
	NK T-cells	$CD3^+$ NKR-$P1A^+$ (%)	3.98	3.86	3.35	3.28	3.76	3.62	3.48	3.63
	Activated T-cells	$CD3^-$ NKR-$P1A^+$ (%)	1.87	2.27	1.77	2.29	1.87	2.59	1.93	2.34
	NK cells	$CD3^-$ NKR-$P1A^+$ (%)	11.02	14.48	10.02	14.10	9.41	12.10	9.71	13.65
	B cells	$CD45RA^+$ cells (%)	29.86	23.11	30.46	27.82	26.88	25.28	28.70	27.69

Data are presented as mean ± SD. * $p \leq 0.05$ compared to vehicle.
Lymphocyte phenotyping was only done in the 14-day follow-up toxicology animals, before the first and after the last treatment administration.

Table S5. Plasma cytokine levels in rats.

Gender	Cytokines	14 day follow-up (n=10 per group)	
		Vehicle (n=10)	400 mg/kg/day Adrecizumab (n=10)
Male	GM-CSF	BQL	BQL
	IFN-γ	BQL	BQL
	IL-10	BQL	BQL
	IL-12	724.02 ± 181.20	693.54 ± 195.57
	IL-1β	BQL	BQL
	IL-2	93.38 ± 20.24	77.59 ± 31.94
	IL-4	BQL	BQL
	IL-6	BQL	BQL
	TNF-α	BQL	BQL
Female	GM-CSF	BQL	BQL
	IFN-γ	BQL	BQL
	IL-10	BQL	BQL
	IL-12	491.26 ± 169.78	486.49 ± 166.74
	IL-1β	BQL	BQL
	IL-2	52.09 ± 33.44	71.38 ± 39.20
	IL-4	BQL	BQL
	IL-6	BQL	BQL
	TNF-α	BQL	BQL

Data are presented as mean ± SD. * p ≤ 0.05 compared to vehicle.
Abbreviations: BQL (below quantification limit); GM-CSF (granulocyte-macrophage colony-stimulating factor); IFN-γ (interferon gamma); IL (interleukin); n (number of animals); TNF-α (tumor necrosis factor alpha).

Table S6. Macroscopic abnormalities in rats.

Gender	Organ info		14 day follow-up (n=10 per group)				Recovery animals (n=5 per group)	
			Vehicle	100 mg/kg/day Adrecizumab	200 mg/kg/day Adrecizumab	400 mg/kg/day Adrecizumab	Vehicle	400 mg/kg/day Adrecizumab
Male	General condition	Normal	n=10 rats	n=10 rats	n=10 rats	n=10 rats	n=5 rats	n=5 rats
	No abnormalities detected		n=9 rats	n=5 rats	n=7 rats	n=7 rats	n=2 rats	n=3 rats
	Lung	Red areas	-	n=1 rats	n=2 rats	n=1 rats	n=1 rats	n=1 rats
		Red foci	-	n=3 rats	-	n=2 rats	n=2 rats	n=1 rats
	Liver	Hepato-diaphragmatic nodule	n=1 rats	-	-	-	-	-
	Kidney	Right dilated pelvix	-	n=2 rats	n=1 rats	-	-	-
		Left dilated pelvix	-	n=1 rats	-	-	-	-
	Testes	Small, unilateral (left)	-	n=1 rats	-	-	-	-
	Adrenals	Enlarged	-	-	-	-	-	-
Female	General condition	Normal	n=10 rats	n=10 rats	n=10 rats	n=10 rats	n=5 rats	n=5 rats
	No abnormalities detected		n=8 rats	n=8 rats	n=9 rats	n=9 rats	n=4 rats	n=4 rats
	Lung	Red areas	n=1 rats	n=1 rats	-	-	-	-
		Red foci	-	-	-	n=1 rats	-	-
	Liver	Hepato-diaphragmatic nodule	-	n=1 rats	-	-	-	-
	Kidney	Right dilated pelvix	-	-	-	-		-
		Left dilated pelvix	-	-	-	-		-
	Ovaries	Cyst, unilateral	n=1 rats	-	n=1 rats	-	-	-
	Cervical lymph node	Reddish discoloration	-	-	-	-	-	n=1 rats
	Adrenals	Enlarged	-	-	-	-	n=1 rats	-
	Abdominal fat tissue	Mass in lateral uterine ligamentum	-	-	-	-	n=1 rats	-

Abbreviations: n (number of animals).

Table S7. Absolute and relative organ weights in rats.

Gender	Organ		14 day follow-up (n=10 per group)				42-day follow-up (n=5 per group)	
			Vehicle	100 mg/kg/day Adrecizumab	200 mg/kg/day Adrecizumab	400 mg/kg/day Adrecizumab	Vehicle	400 mg/kg/day Adrecizumab
Male	Adrenals (gram)	Absolute	0.075 ± 0.005	0.078 ± 0.007	0.071 ± 0.011	0.075 ± 0.009	0.059 ± 0.018	0.071 ± 0.006
		Relative to BW	0.021 ± 0.001	0.021 ± 0.002	0.019 ± 0.003	0.022 ± 0.003	0.013 ± 0.004	0.015 ± 0.002
	Brain (gram)	Absolute	2.00 ± 0.082	1.97 ± 0.059	1.94 ± 0.084	1.97 ± 0.074	2.10 ± 0.058	2.13 ± 0.091
		Relative to BW	0.565 ± 0.036	0.539 ± 0.042	0.529 ± 0.054	0.565 ± 0.036	0.451 ± 0.046	0.457 ± 0.058
	Heart (gram)	Absolute	1.09 ± 0.096	1.13 ± 0.130	1.15 ± 0.126	1.05 ± 0.100	1.34 ± 0.131	1.32 ± 0.089
		Relative to BW	0.308 ± 0.025	0.307 ± 0.023	0.312 ± 0.035	0.301 ± 0.026	0.287 ± 0.020	0.283 ± 0.011
	Kidneys (gram)	Absolute	2.88 ± 0.199	2.71 ± 0.149	2.74 ± 0.172	2.75 ± 0.199	2.98 ± 0.322	3.04 ± 0.271
		Relative to BW	0.811 ± 0.058	0.743 ± 0.065	0.746 ± 0.079	0.787 ± 0.057	0.637 ± 0.029	0.650 ± 0.045
	Liver (gram)	Absolute	11.71 ± 0.749	11.49 ± 1.276	10.42 ± 1.098	11.12 ± 1.223	12.05 ± 1.718	12.11 ± 2.400
		Relative to BW	3.298 ± 0.209	3.136 ± 0.252	2.835 ± 0.334*	3.181 ± 0.367	2.565 ± 0.164	2.562 ± 0.329
	Prostate (gram)[A]	Absolute	2.01 ± 0.317	2.21 ± 0.308	2.41 ± 0.353	2.21 ± 0.366	2.83 ± 0.412	2.70 ± 0.390
		Relative to BW	0.566 ± 0.082	0.603 ± 0.083	0.651 ± 0.076	0.632 ± 0.105	0.610 ± 0.116	0.582 ± 0.115
	Spleen (gram)	Absolute	0.894 ± 0.112	0.815 ± 0.150	0.740 ± 0.103*	0.768 ± 0.063*	0.840 ± 0.233	0.871 ± 0.073
		Relative to BW	0.252 ± 0.033	0.222 ± 0.033	0.202 ± 0.036*	0.220 ± 0.021	0.177 ± 0.038	0.186 ± 0.015
	Testes (gram)	Absolute	3.71 ± 0.316	3.39 ± 0.290	3.45 ± 0.370	3.67 ± 0.063	3.67 ± 0.391	3.66 ± 0.338
		Relative to BW	1.046 ± 0.085	0.929 ± 0.113	0.940 ± 0.125	1.051 ± 0.099	0.788 ± 0.099	0.784 ± 0.088
	Epididymides (gram)	Absolute	1.12 ± 0.160	1.07 ± 0.194	1.20 ± 0.144	1.17 ± 0.151	1.54 ± 0.109	1.49 ± 0.109
		Relative to BW	0.313 ± 0.034	0.294 ± 0.052	0.326 ± 0.036	0.335 ± 0.046	0.332 ± 0.043	0.319 ± 0.043
	Thymus (gram)	Absolute	0.496 ± 0.105	0.534 ± 0.107	0.476 ± 0.096	0.476 ± 0.118	0.380 ± 0.062	0.482 ± 0.088
		Relative to BW	0.139 ± 0.027	0.146 ± 0.028	0.130 ± 0.027	0.136 ± 0.031	0.081 ± 0.011	0.102 ± 0.016*

Table S7. continued.

Gender	Organ		14 day follow-up (n=10 per group)				42-day follow-up (n=5 per group)	
			Vehicle	100 mg/kg/day Adrecizumab	200 mg/kg/day Adrecizumab	400 mg/kg/day Adrecizumab	Vehicle	00 mg/kg/day Adrecizumab
Female	Adrenals (gram)	Absolute	0.092 ± 0.010	0.088 ± 0.013	0.097 ± 0.015	0.097 ± 0.013	0.112 ± 0.023	0.096 ± 0.013
		Relative to BW	0.038 ± 0.004	0.037 ± 0.005	0.040 ± 0.005	0.039 ± 0.005	0.039 ± 0.007	0.033 ± 0.003
	Brain (gram)	Absolute	1.82 ± 0.092	1.85 ± 0.031	1.83 ± 0.029	1.86 ± 0.066	1.91 ± 0.094	1.89 ± 0.068
		Relative to BW	0.754 ± 0.063	0.773 ± 0.035	0.758 ± 0.041	0.753 ± 0.060	0.678 ± 0.057	0.651 ± 0.044
	Heart (gram)	Absolute	0.813 ± 0.046	0.794 ± 0.053	0.820 ± 0.051	0.838 ± 0.071	0.917 ± 0.038	0.900 ± 0.153
		Relative to BW	0.336 ± 0.013	0.331 ± 0.019	0.338 ± 0.016	0.338 ± 0.025	0.325 ± 0.027	0.308 ± 0.035
	Kidneys (gram)	Absolute	1.81 ± 0.164	1.74 ± 0.131	1.80 ± 0.151	1.83 ± 0.155	1.98 ± 0.196	1.94 ± 0.215
		Relative to BW	0.745 ± 0.060	0.725 ± 0.043	0.742 ± 0.045	0.738 ± 0.038	0.698 ± 0.033	0.663 ± 0.028
	Liver (gram)	Absolute	7.36 ± 0.768	7.48 ± 0.383	7.63 ± 0.800	8.23 ± 0.794*	8.41 ± 0.972	8.62 ± 1.077
		Relative to BW	3.036 ± 0.260	3.123 ± 0.159	3.136 ± 0.192	3.317 ± 0.253*	2.966 ± 0.217	2.948 ± 0.150
	Ovaries (gram)	Absolute	0.156 ± 0.029	0.173 ± 0.032	0.162 ± 0.022	0.152 ± 0.015	0.200 ± 0.012	0.189 ± 0.039
		Relative to BW	0.064 ± 0.011	0.072 ± 0.012	0.067 ± 0.008	0.061 ± 0.004	0.071 ± 0.006	0.064 ± 0.009
	Spleen (gram)	Absolute	0.623 ± 0.101	0.593 ± 0.111	0.626 ± 0.077	0.626 ± 0.070	0.654 ± 0.078	0.619 ± 0.103
		Relative to BW	0.257 ± 0.039	0.246 ± 0.037	0.258 ± 0.033	0.253 ± 0.031	0.231 ± 0.018	0.213 ± 0.040
	Thymus (gram)	Absolute	0.404 ± 0.079	0.425 ± 0.084	0.394 ± 0.087	0.404 ± 0.064	0.412 ± 0.047	0.417 ± 0.048
		Relative to BW	0.166 ± 0.028	0.176 ± 0.030	0.163 ± 0.034	0.163 ± 0.026	0.146 ± 0.021	0.143 ± 0.018
	Uterus (gram)	Absolute	0.529 ± 0.165	0.563 ± 0.260	0.532 ± 0.183	0.432 ± 0.088	0.501 ± 0.125	0.617 ± 0.241
		Relative to BW	0.218 ± 0.067	0.234 ± 0.102	0.221 ± 0.082	0.173 ± 0.030	0.177 ± 0.044	0.208 ± 0.064

[A] Including seminal vesicle and coagulating gland.
* $p \leq 0.05$ compared to vehicle.
Abbreviations: bodyweight (BW); n (number of animals).

Table S8A. Histopathological findings in male rats.

Histopathological findings		14 day follow-up (n=10 per group)				Recovery animals (n=5 per group)	
		Vehicle	100 mg/kg/day	200 mg/kg/day	400 mg/kg/day	Vehicle	400 mg/kg/day
Liver	Inflammatory cell infiltration, focal/perivascular/portal	1 (n=4)	1 (n=4)	1 (n=7) 2 (n=2)	1 (n=5)	1 (n=1)	1 (n=2)
	Hepato-diaphragmatic nodule	1 (n=1)	-	-	-	-	-
Adrenals	Vacuolisation, cortex	-	-	M (n=1)	-	1 (n=1)	-
Spleen	Extramedullaris hemopoesis	1 (n=2)	1 (n=2)	1 (n=3)	1 (n=1)	1 (n=4) 2 (n=1)	1 (n=5)
Kidney	Cortical tubular degeneration, focal	-	-	-	1 (n=2) 2 (n=1)	-	-
	Tubular basophilia, focal	1 (n=1)	-	-	1 (n=2)	-	1 (n=1)
	Tubular casts	-	1 (n=1)	-	1 (n=1)		
	Pelvis dilatation, bilateral	2 (n=2)	2 (n=1) 3 (n=1)	-	-	-	-
	Pelvis dilatation, unilateral	-	-	2 (n=1)	-	2 (n=2)	-
Pancreas	Atrophy, focal	-	-	2 (n=1)	1 (n=1)	-	-
	Lymphoid cell infiltration, focal	-	-	-	1 (n=2)	-	-
Lung	Aggregats of alveolar macrophages, focal	-	-	1 (n=2)	1 (n=2)	-	-
	Hematoidin crystals, alveolar spaces, focal	-	1 (n=1)	1 (n=1)	1 (n=1)	-	-
	Haemorrhages, focal/multifocal	1f (n=1)	1f (n=3) 1m (n=1) 2m (n=1)	1m (n=2) 1f (n=3)	1f (n=5)	1f (n=1)	1m (n=2)
	Inflammatory cell infiltration, perivascular/peribroncial	-	1 (n=1)	3 (n=1)	-	-	-
	Lymphoid cell infiltration, focal	1 (n=1)	-	-	-	-	-
	Osseous metaplasia	-	1 (n=1)	-	-	-	1 (n=1)
Eye	Fully desorganised eyeball, with reparative tissue	-	-	-	4 (n=3)	1 (n=1)	1 (n=1)
	Inflammatory cell infiltration, periocular, unilateral	2 (n=1)	-	-	-	-	-
	Retinal folding, focal				1 (n=1)	-	-
Harderian gland	Lymphocytic infiltration	1 (n=1)	1 (n=1)	-	-	-	-
	Basophilia/dilated acini/atrophy/haemorrhage/inflammation, unilateral	1 (n=2) 2 (n=1)	-	1 (n=2) 2 (n=1)	1 (n=2) 2 (n=1) 3 (n=2)	-	2 (n=1)
	Basophili/dilated acini/atrophy/haemorrhage/inflammation, bilateral	3 (n=1)	-	-	-	-	-
Thymus	Tingible body macrophages, cortex	1 (n=1)	-	1 (n=2)	1 (n=1)	1 (n=1)	1 (n=1)
	Haemorrhage, focal	-	-	-	-	-	-
Thyroid	Cyst	-	-	-	1 (n=1)	-	-
	Increased lymphoid follicles	-	-	-	-	2 (n=1)	-

Table S8A. continued.

Histopathological findings		14 day follow-up (n=10 per group)				Recovery animals (n=5 per group)	
		Vehicle	100 mg/kg/day	200 mg/kg/day	400 mg/kg/day	Vehicle	400 mg/kg/day
Prostate	Atrophy, focal	1 (n=2)	1 (n=2)	1 (n=2) 2 (n=1)	1 (n=2) 2 (n=1)	-	-
	Acinar dilatation	-	-	3 (n=1)	-	-	-
	Lymphocytic infiltration, diffuse	1 (n=1)	-	1 (n=1)	-	-	-
Testes	Tubular atrophy, unilateral, multifocal	-	2 (n=1)	-	-	-	-
	Tubular atrophy, unilateral, focal	-	-	1 (n=2)			
	Desorganisation/degeneration of tubular semiferous epithelium, focal	-	-	1 (n=1)	-	-	-
	Cystic dilatation, rete testis	-	-	1 (n=1)	-	-	-
Mammary		N (n=3)	N (n=2)	N (n=2)	N (n=3)	N (n=5)	N (n=4)
Heart	Lympho-histiocytic infiltration, focal/multifocal	1f (n=4) 2f (n=1)	1m (n=1) 1f (n=1)	1f (n=2)	-	-	-
	Thickened endocardium, right ventricle	2 (n=1)	-	-	-	-	-
	Haemorrhage, subpericardial	-	-	-	-	-	2 (n=1)
Skeletal muscle	Necrosis, focal fibrillar	1 (n=1)	-	1 (n=1)	-	-	-
Tail/site of admin.	Haemorrhage, perivascular	1 (n=1) 2 (n=1)	1 (n=3) 2 (n=1)	1 (n=2) 2 (n=3)	1 (n=4) 2 (n=2)	-	-
	Inflammatory cell infiltration, perivascular	1 (n=1) 2 (n=1)	1 (n=1) 2 (n=1)	1 (n=2)	1 (n=5)	1 (n=1)	-
	Blood clot/fibrin precipitate, vein lumen	-	-	1 (n=1)	1 (n=1)	-	-
	Fibrin precipitate, perivascular	1 (n=1)	-	1 (n=3)	1 (n=3)	-	-
	Thickened endothelium	1 (n=1)	1 (n=1)	1 (n=1)	-	-	-
	Dilated vein, unilateral	2 (n=1)	2 (n=1)	2 (n=1)	2 (n=4)	2 (n=1)	-
	Dilated vein, bilateral	2 (n=1)	2 (n=3)	2 (n=1)	-	2 (n=2)	-
Mesenteric lymph node		-	-	-	M (n=1)	-	-
Cervical lymph node	Erythrocytosis/erythrocytophagia	1 (n=1)	1 (n=2) 2 (n=1)	1 (n=1)	2 (n=1)	-	-
Pituitary	Cyst	2 (n=2)	-	-	-	-	-

Abbreviations: 1 (minimal); 2 (mild); 3 (moderate); 4 (severe alteration); f (focal); m (multifocal); M (missing); n (number of affected animals); N = not present.

Table S8B. Histopathological findings in female rats.

Histopathological findings		14 day follow-up (n=10 per group)				Recovery animals (n=5 per group)	
		Vehicle	100 mg/kg/day	200 mg/kg/day	400 mg/kg/day	Vehicle	400 mg/kg/day
Liver	Inflammatory cell infiltration, focal/perivascular/portal	1 (n=3)	1 (n=7)	1 (n=4)	1 (n=2)	-	1 (n=1)
	Hepato-diaphragmatic nodule	-	-	-	-	-	-
Adrenals	Lymphocytic infiltration, focal, cortex	-	-	-	1 (n=1)	2 (n=1)	1 (n=1) 2 (n=2)
	Vacuolisation, cortex	-	-	-	-	-	-
Spleen	Extramedullaris hemopoesis	1 (n=4) 2 (n=3)	1 (n=6) 2 (n=2)	1 (n=6)	1 (n=3) 2 (n=1)	1 (n=2)	1 (n=2)
Kidney	Cortical tubular degeneration, focal	1 (n=1)	-	-	-	-	-
	Tubular basophilia, focal	-	-	-	-	-	-
	Tubular casts	-	-	-	-	-	-
	Tubular dilatation	-	-	-	-	1 (n=1)	-
	Pelvis dilatation, bilateral	-	-	-	-	-	-
	Pelvis dilatation, unilateral	-	-	-	-	-	-
	Mineralisation, cortical	-	1 (n=1)	-	1 (n=1)	-	1 (n=1)
	Mineralisation, papillary	-	-	-	-	-	1 (n=1)
	Cyst, cortical	2 (n=1)	2 (n=1)	-	2 (n=1)	-	-
	Hyperplasia, transitional epithelium, focal	-	2 (n=1)	-	-	-	-
	Inflammatory cell infiltration, suburothelial	-	1 (n=1)	-	-	2 (n=1)	-
Pancreas	Atrophy, focal	-	-	-	-	-	-
	Lymphoid cell infiltration, focal	-	-	-	-	-	-
Lung	Aggregats of alveolar macrophages, focal	-	-	-	1 (n=1)	-	-
	Hematoidin crystals, alveolar spaces, focal	1 (n=1)	-	-	-	-	-
	Haemorrhages, focal/multifocal	1f (n=2)	1f (n=1)	-	1f (n=5)	-	1m (n=1)
	Inflammatory cell infiltration, perivascular/peribroncial	-	-	-	-	-	-
	Inflammatory cell infiltration, perivascular, eosinophils	1 (n=1) 2 (n=1)	1 (n=1)	-	2 (n=1)	-	-
	Lymphoid cell infiltration, focal	-	-	-	-	-	-
	Osseous metaplasia	-	-	-	-	1 (n=1)	-

Table S8B. Continued.

Histopathological findings		14 day follow-up (n=10 per group)				Recovery animals (n=5 per group)	
		Vehicle	100 mg/ kg/day	200 mg/ kg/day	400 mg/ kg/day	Vehicle	400 mg/ kg/day
Eye	Granulomatous inflammation/fibrosis, unilateral	-	-	-	-	-	-
	Fully desorganised eyeball, with reparative tissue	-	-	-	-	-	-
	Inflammatory cell infiltration, periocular, unilateral	-	-	-	-	-	-
Harderian gland	Lymphocytic infiltration	-	-	1 (n=1)	-	-	-
	Basophilia/dilated acini/ atrophy/haemorrhage/ inflammation, unilateral	1 (n=1) 2 (n=1)	1 (n=1)	1 (n=1) 2 (n=1)	-	-	2 (n=1)
	Basophili/dilated acini/ atrophy/haemorrhage/ inflammation, bilateral	-	-	-	-	-	-
Thymus	Perithymic Lnn	-	-	-	-	-	-
	Erythrocytosis/ erythrocytophagia	-	-	-	2 (n=1)	-	-
	Tingible body macrophages, cortex	1 (n=4)	1 (n=3)	1 (n=3) 2 (n=1)	1 (n=2)	-	-
	Haemorrhage, focal	-	-	-	-	-	-
Thyroid	Cyst	-	-	-	-	-	-
	Increased lymphoid follicles	1 (n=1)	-	-	-	-	-
Vagina	Inflammatory cell infiltration	-	-	2 (n=1)	-	-	-
Ovaries	Cyst, unilateral	-	-	1 (n=1)	-	-	-
Mammary		-	M (n=1)	-	-	-	-
Heart	Lympho-histiocytic infiltration, focal/ multifocal	1f (n=1)	-	-	-	-	-
	Thickened endocardium, right ventricle	1 (n=1)	-	-	-	-	-
	Haemorrhages	1 (n=1)	1 (n=1)	1 (n=4)	-	-	-
Skeletal muscle	Necrosis, focal fibrillar	1 (n=1)	-	1 (n=1)	1 (n=1)	-	-
	Inflammatory cell infiltration, focal	1 (n=1)	-	-	1 (n=1)	-	-

Table S8B. Continued.

Histopathological findings		14 day follow-up (n=10 per group)				Recovery animals (n=5 per group)	
		Vehicle	100 mg/ kg/day	200 mg/ kg/day	400 mg/ kg/day	Vehicle	400 mg/ kg/day
Tail/site of admin.	Haemorrhage, perivascular	1 (n=3) 2 (n=1)	1 (n=2)	1 (n=2) 2 (n=1)	1 (n=4) 2 (n=1)	-	-
	Inflammatory cell infiltration, perivascular	1 (n=2)	-	1 (n=2) 2 (n=4)	1 (n=5) 2 (n=1)	-	-
	Blood clot/fibrin precipitate, vein lumen	-	-	2 (n=1)	1 (n=1)	-	-
	Fibrin precipitate, perivascular	1 (n=2)	1 (n=1)	1 (n=1)	1 (n=1)	-	-
	Hair embolus	-	-	-	-	-	-
	Recanalisation	-	-	-	-	-	1 (n=1)
	Thickened endothelium	1 (n=1)	-	-	1 (n=1)	-	-
	Dilated vein, unilateral	2 (n=1)	2 (n=1)	-	-	-	-
	Dilated vein, bilateral	-	-	-	-	2 (n=3)	2 (n=2)
Cervical lymph node	Erythrocytosis/ erythrocytophagia	1 (n=3)	-	1 (n=1)	1 (n=1) 2 (n=1)	-	-
Pituitary	Cyst	-	-	-	-	-	-
Femur		M (n=1)	-	-	-	-	-
Oesophagus/ trachea		M (n=1)	-	-	M (n=1)	-	-

Abbreviations: 1 (minimal); 2 (mild); 3 (moderate); 4 (severe alteration); f (focal); m (multifocal); M (missing); n (number of affected animals); N = not present.

Table S9. Toxicokinetic parameters in rats.

Gender	Parameter	100 mg/kg/day Adrecizumab		200 mg/kg/day Adrecizumab		400 mg/kg/day Adrecizumab	
		Day 1	Day 14	Day 1	Day 14	Day 1	Day 14
Male	T_{max} (h)	0.083	0.083	0.25	0.083	0.25	0.083
	C_{max} (µg/mL)	2250	3490	4540	5870	7440	9240
	$C_{max/dose}$ (kg* µg/mL/mg)	25.5	34.9	22.7	29.4	18.6	23.1
	T_{last} (h)	24	24	24	24	24	24
	$C_{last\ (t=24h)}$ µg/mL)	771	1640	1300	3290	2590	4730
	AUC_{0-24h} (h* µg/mL)	27000	46600	47800	94100	107000	142000
	$AUC_{0-24h/dose}$ (h*kg* µg/mL/mg)	270	466	239	471	268	355
	R_{acc}	-	1.73	-	1.97	-	1.33
Female	T_{max} (h)	0.25	0.083	0.25	0.5	0.25	0.083
	C_{max} (µg/mL)	2280	3660	4550	5190	8440	10100
	$C_{max/dose}$ (kg* µg/mL/mg)	22.8	36.6	22.8	26.0	21.1	25.3
	T_{last} (h)	24	24	24	24	24	24
	$C_{last\ (t=24h)}$ µg/mL)	804	1680	1500	3070	2790	5440
	AUC_{0-24h} (h* µg/mL)	26400	48900	54300	86800	10900	150000
	$AUC_{0-24h/dose}$ (h*kg* µg/mL/mg)	264	489	272	434	273	375
	R_{acc}	-	1.85	-	1.60	-	1.38

Data are presented as mean

Table S10. Clinical signs in Beagle dogs (n=3).

Study day	1		2	3		4	5		6	7	8		9	10	11	12	13	14
Treatment	Treatment 0 mg/kg			Treatment 2 mg/kg			Treatment 10 mg/kg				Treatment 50 mg/kg							
	Before	After		Before	After		Before	After			Before	After						
NOA	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Weakness	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Decreased activity	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lying position preferred	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vomitus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spontaneous defecation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spontaneous urination	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Study day	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29		30
Treatment															Treatment 10 mg/kg		
															Before	After	
NOA	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	0	0
Weakness	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3[A]	0
Decreased activity	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2[B]	0
Lying position preferred	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
Vomitus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2[C]	0
Spontaneous defecation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2[D]	0
Spontaneous urination	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1[E]	0

Abbreviations: NOA (no observable abnormality); n (number of animals).

[A] Severe in 1 animal (10-50 min after administration), moderate in 2 (10-30 min after administration).

[B] Moderate in both animals.

[C] One animal at 15 and 55 min after treatment administration, another animal 2 hours after administration.

[D] One animal 30 min after administration, another animal just after.

[E] Just after treatment administration.

Table S11A. Clinical signs in cynomolgus monkeys.

Sex	Dose group (mg/kg/day)	No. of animals	Clinical sign in no. of animals	Study day														
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Male	0	n=5[A]	NOA	0	0	0	1	1	1	3	3	3	5	5	5	5	0	0
			Bruising	5	5	5	4	4	4	2	2	2	0	0	0	0	5	5
			Feces	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
	25	n=3	NOA	1	0	0	0	0	0	3	3	3	3	3	3	3	0	0
			Abnormal colour	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
			Abrasion	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			Bruising	2	3	3	3	3	3	0	0	0	0	0	0	0	3	3
			Hair loss	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	50	n=3	NOA	0	0	0	0	0	0	1	3	2	2	2	2	3	0	0
			Bruising	3	3	3	3	3	3	0	0	1	1	1	1	0	3	3
			Hair loss	1	1	2	2	2	2	2	0	0	0	0	0	0	0	0
			Scab/crust	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	100	n=5[A]	NOA	1	1	0	0	0	0	1	2	2	3	3	3	1	0	0
			Bruising	4	4	5	5	5	5	3	1	1	0	0	0	2	5	5
			Discharge	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			Hair loss	3	3	3	3	3	2	2	2	2	2	2	2	2	2	2
			Scab/crust	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1
Female	0	n=5[A]	NOA	0	0	0	0	0	0	1	2	1	2	2	2	2	0	0
			Abnormal colour	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1
			Bruising	5	5	5	5	5	5	4	3	3	2	2	2	2	4	4
			Discharge	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0
			Hair loss	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0
			Scab/crust	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
	25	n=3	NOA	0	0	0	0	0	0	1	2	2	2	2	2	2	0	0
			Abnormal colour	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
			Bruising	3	3	3	3	3	3	1	0	0	0	0	0	0	3	3
			Hair loss	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1
			Scab/crust	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
	50	n=3	NOA	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0
			Abnormal colour	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
			Bruising	3	3	3	3	3	3	1	1	1	1	1	1	1	0	3
			Discharge	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
			Feces	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
			Hair loss	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2
	100	n=5[A]	NOA	0	0	0	0	0	0	3	2	1	3	2	4	4	0	0
			Abnormal colour	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
			Abrasion	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			Bruising	5	5	5	5	5	5	2	1	2	0	0	0	0	4	5
			Discharge	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
			Feces	1	0	0	0	0	0	0	3	1	1	1	0	0	0	0
			Hair loss	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1

[A] Includes the first 14 study days of the 5 recovery animals in the vehicle and 400 mg/kg group.
Abbreviations: NOA (no observable abnormality).

Table S11B. Clinical signs in cynomolgus monkeys (recovery animals).

Sex	Dose group (mg/kg/day)	No. of animals	Clinical sign in no. of animals	Study day														
				16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Male	0	n=2	NOA	0	0	0	0	0	0	1	2	2	2	2	2	2	2	2
			Bruising	2	2	2	2	2	2	1	0	0	0	0	0	0	0	0
			Discharge	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	100	n=2	NOA	0	0	0	0	0	0	1	1	2	2	2	2	2	2	2
			Bruising	2	2	2	2	2	2	1	0	0	0	0	0	0	0	0
			Discharge	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			Hair loss	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
			Scab/crust	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Female	0	n=2	NOA	0	0	0	0	0	0	1	1	2	2	2	2	2	2	2
			Abnormal colour	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
			Bruising	2	2	2	2	2	2	0	0	0	0	0	0	0	0	0
			Discharge	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			Scab/crust	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
	100	n=2	NOA	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
			Bruising	2	2	2	2	0	0	0	0	0	0	0	0	0	0	0
			Hair loss	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Abbreviations: NOA (no observable abnormality; n (number of animals).

Sex	Dose group (mg/kg/day)	No. of animals	Clinical sign in no. of animals	Study day											
				31	32	33	34	35	36	37	38	39	40	41	42
Male	0	n=2	NOA	2	2	2	2	2	2	1	1	1	1	0	1
			Bruising	0	0	0	0	0	0	1	1	1	1	2	1
			Discharge	0	0	0	0	0	0	0	0	0	0	1	0
	100	n=2	NOA	2	2	1	1	1	1	1	1	1	1	0	1
			Bruising	0	0	0	0	0	0	0	0	0	0	1	0
			Discharge	0	0	0	0	0	0	0	0	0	0	1	0
			Hair loss	0	0	1	1	1	1	1	1	1	1	1	1
			Scab/crust	0	0	0	0	0	0	0	0	0	0	0	0
Female	0	n=2	NOA	2	2	2	2	2	2	2	2	2	2	1	2
			Abnormal colour	0	0	0	0	0	0	0	0	0	0	0	0
			Bruising	0	0	0	0	0	0	0	0	0	0	0	0
			Discharge	0	0	0	0	0	0	0	0	0	0	1	0
			Scab/crust	0	0	0	0	0	0	0	0	0	0	0	0
	100	n=2	NOA	1	1	1	1	1	1	1	1	1	1	1	1
			Bruising	0	0	0	0	0	0	0	0	0	0	1	1
			Hair loss	1	1	1	1	1	1	1	1	1	1	1	1

Abbreviations: NOA (no observable abnormality; n (number of animals).

Table S12. Neurological observations in cynomolgus monkeys.

Dose group (mg/kg/day)	No. of animals	Neurological observation	Male		Female	
			Day -10	Day 15	Day -10	Day 15
0	n=5	Stimulus response	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Behaviour	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Gait	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Brachiation	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Posture	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Grasp	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Activity level	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Balance	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Conjugate movement	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Position	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Pupil reactivity	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
25	n=3	Stimulus response	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Behaviour	Normal (n=3)	Normal (n=3)	Head tilt (n=1) Normal (n=2)	Head tilt (n=1) Normal (n=2)
		Gait	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Brachiation	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Posture	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Grasp	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Activity level	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Balance	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Conjugate movement	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Position	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Pupil reactivity	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
50	n=3	Stimulus response	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Behaviour	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Gait	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Brachiation	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Posture	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Grasp	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Activity level	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Balance	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Conjugate movement	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Position	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
		Pupil reactivity	Normal (n=3)	Normal (n=3)	Normal (n=3)	Normal (n=3)
100	n=5	Stimulus response	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Behaviour	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Gait	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Brachiation	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Posture	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Grasp	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Activity level	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Balance	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Conjugate movement	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Position	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)
		Pupil reactivity	Normal (n=5)	Normal (n=5)	Normal (n=5)	Normal (n=5)

Abbreviations: n (number of animals).

Table S13. Electrocardiographic parameters in cynomolgus monkeys.

Dose group (mg/kg/day)	No. of animals	ECG parameter	Male		Female	
			Day -9	Day 14	Day -9	Day 14
0	n=5	RR (msec)	325 ± 15	372 ± 40	335 ± 46	353 ± 42
		HR (bpm)	185 ± 8	163 ± 18	182 ± 26	172 ± 20
		PR (msec)	73 ± 5	76 ± 6	77 ± 6	79 ± 8
		QRS (msec)	34 ± 3	33 ± 3	33 ± 2	33 ± 4
		QT (msec)	197 ± 6	214 ± 11	200 ± 20	207 ± 19
		QTc (msec)	347 ± 12	351 ± 9	346 ± 17	348 ± 17
25	n=3	RR (msec)	314 ± 28	354 ± 20	319 ± 31	351 ± 65
		HR (bpm)	192 ± 17	169 ± 10	189 ± 19	175 ± 32
		PR (msec)	68 ±5	68 ± 6	72 ± 2	73 ± 1
		QRS (msec)	32 ± 1	32 ± 4	34 ± 2	35 ± 2
		QT (msec)	193 ± 11	204 ± 10	200 ± 15	209 ± 21
		QTc (msec)	343 ± 7	343 ± 11	354 ± 12	353 ± 9
50	n=3	RR (msec)	357 ± 18	331 ± 31	302 ± 24	322 ± 33
		HR (bpm)	168 ± 9	183 ± 18	200 ± 17	188 ± 20
		PR (msec)	74 ± 9	68 ± 10	166 ± 13	67 ± 11
		QRS (msec)	35 ± 5	32 ± 2	34 ± 4	33 ± 4
		QT (msec)	217 ± 13	202 ± 12	192 ± 11	202 ± 9
		QTc (msec)	362 ± 13	352 ± 10	349 ± 9	356 ± 5
100	n=5	RR (msec)	343 ± 55	383 ± 62	363 ± 27	359 ± 38
		HR (bpm)	178 ± 28	160 ± 27	166 ± 13	169 ± 18
		PR (msec)	75 ± 8	76 ± 4	84 ± 7	79 ± 12
		QRS (msec)	34 ± 2	34 ± 2	34 ± 4	36 ± 5
		QT (msec)	206 ± 23	217 ± 24	211 ± 6	213 ± 17
		QTc (msec)	35 ± 15	351 ± 18	351 ± 8	355 ± 15

Data are presented as mean ± SD .
Abbreviations: bpm (beats per minute); HR (heart rate); msec (millisecond); QTc (corrected QT-time, using Bazett's formula).

Table S14. Blood pressure and respiratory rate in cynomolgus monkeys.

Dose group (mg/kg/day)	No. of animals	Parameter	Male		Female	
			Day -9	Day 14	Day -9	Day 14
0	5	SBP (mmHg)	126 ± 26	140 ± 40	137 ± 13	145 ± 31
		DBP (mmHg)	64 ± 8	62 ± 11	77 ± 4	82 ± 21
25	3	SBP (mmHg)	114 ± 2	109 ± 6	135 ± 18	131 ± 3
		DBP (mmHg)	58 ± 5	62 ± 13	84 ± 10	62 ± 5
50	3	SBP (mmHg)	109 ± 11	116 ± 17	136 ± 37	137 ± 39
		DBP (mmHg)	68 ± 14	61 ± 6	81 ± 29	77 ± 14
100	5	SBP (mmHg)	128 ± 12	134 ± 21	133 ± 26	149 ± 30
		DBP (mmHg)	74 ± 18	78 ± 11	79 ± 14	94 ± 14
0	5	Respiratory rate (rate/min)	61 ± 21	54 ± 22	55 ± 16	52 ± 16
25	3	Respiratory rate (rate/min)	72 ± 32	65 ± 26	48 ± 7	59 ± 10
50	3	Respiratory rate (rate/min)	67 ± 6	61 ± 8	60 ± 17	52 ± 12
100	5	Respiratory rate (rate/min)	41 ± 3	46 ± 7	43 ± 10	43 ± 12

Data are presented as mean ± SD.
Abbreviations: DBP (diastolic blood pressure); mmHg (millimetres mercury); SBP (systolic blood pressure).

Table S15. Urinalysis in cynomolgus monkeys.

Dose group (mg/kg/day)	ECG parameter	Male			Female		
		Day -6	Day 15	Day 41 (recovery animals)	Day -6	Day 15	Day 41 (recovery animals)
0 (n=5)	Specific gravity	1.017 ± 0.005	1.022 ± 0.012	1.019 ± 0.006	1.021 ± 0.008	1.021 ± 0.015	1.020 ± 0.011
	Volume (mL)	58.0 ± 17.9	34.8 ± 27.4	28.0 ± 9.9	60.0 ± 33.9	118.2 ± 128.4	74.5 ± 62.9
	Creatinine (mg/dL)	69.6 ± 20.1	105.3 ± 45.0	68.3 ± 29.3	81.81 ± 16.0	95.0 ± 82.0	96.7 ± 61.7
	Sodium (mEq/L)	33.6 ± 17.7	40.1 ± 9.6	20.4 ± 2.3	55.8 ± 30.5	39.5 ± 14.7	49.7 ± 18.8
	Potassium (mEq/L)	83.9 ± 12.1	66.7 ± 47.3	75.3 ± 18.5	94.5 ± 33.4	61.8 ± 49.6	85.3 ± 61.7
	Chloride (mEq/L)	40.7 ± 14.9	66.1 ± 43.8	56.8 ± 12.0	50.7 ± 13.3	57.8 ± 41.98	62.0 ± 33.2
	Colour	Yellow (n=5)	Yellow (n=5)	Yellow (n=2)	Red (n=1) Yellow (n=4)	Yellow (n=5)	Yellow (n=2)
	Clarity	Clear (n=5)	Sl cloudy (n=1) Cloudy (n=2) Clear (n=2)	Sl cloudy (n=2)	Sl cloudy (n=1) Clear (n=4)	Sl cloudy (n=2) Cloudy (n=3)	Cloudy (n=2)
	Nitrates[A]	Positive (n=1) Negative (n=4)	Positive (n=5)	Negative (n=2)	Negative (n=5)	Negative (n=5)	Negative (n=2)
	Glucose[A]	Negative (n=5)	Negative (n=5)	Negative (n=2)	Negative (n=5)	Negative (n=5)	Negative (n=2)
	Ketones[A]	1+ (n=1) Negative (n=4)	1+ (n=1) 2+ (n=1) Negative (n=3)	Negative (n=2)	Negative (n=5)	1+ (n=3) 2+ (n=1) Negative (n=1)	Trace (n=1) Negative (n=1)
	Blood[A]	Negative (n=5)	Trace (n=2) 2+ (n=2) Negative (n=1)	Negative (n=2)	Trace (n=1) 1+ (n=1) 3+ (n=1) Negative (n=2)	Trace (n=2) 1+ (n=1) 2+ (n=2)	2+ (n=1) Negative (n=1)
	Protein[A]	Negative (n=5)	1+ (n=1) Negative (n=4)	Negative (n=2)	Trace (n=2) Negative (n=3)	Trace (n=1) 1+ (n=1) Negative (n=3)	Trace (n=1) Negative (n=1)
	Bilirubin[A]	Negative (n=5)	Negative (n=5)	Negative (n=2)	Negative (n=5)	Negative (n=5)	Negative (n=2)
	pH[A]	8.5 (n=5)	7.0 (n=1) 8.0 (n=2) 8.5 (n=2)	8.5 (n=1) >=9.0 (n=1)	8.0 (n=1) 8.5 (n=4)	7.0 (n=2) 7.5 (n=1) 8.5 (n=2)	8.5 (n=1) >=9.0 (n=1)
	Leukocytes[A]	Negative (n=5)	2+ (n=1) Negative (n=4)	Negative (n=2)	Trace (n=1) 1+ (n=2) Negative (n=3)	1+ (n=3) Negative (n=3)	Trace (n=1) Negative (n=1)
	Urobilirubin (EU/dL)	0.2 (n=5)	0.2 (n=4) 1.0 (n=1)	0.2 (n=2)	0.2 (n=5)	0.2 (n=4) 1.0 (n=1)	0.2 (n=2)
25 (n=3)	Specific gravity	1.020 ± 0.010	1.021 ± 0.015		1.026 ± 0.007	1.021 ± 0.017	
	Volume (mL)	76.7 ± 47.3	148.0 ± 162.2		46.7 ± 11.5	61.7 ± 39.0	
	Creatinine (mg/dL)	70.7 ± 25.9	80.2 ± 74.7		117.5 ± 45.0	98.6 ± 55.6	
	Sodium (mEq/L)	26.0 ± 5.1	13.5 ± 3.2		28.6 ± 9.9	27.1 ± 10.8	
	Potassium (mEq/L)	89.5 ± 43.1	72.9 ± 55.3		104.3 ± 41.8	31.5 ± 25.3	
	Chloride (mEq/L)	38.9 ± 14.5	42.4 ± 14.1		48.2 ± 19.3	44.3 ± 24.4	
	Colour	Yellow (n=3)	Orange (n=1) Yellow (n=2)		Yellow (n=3)	Yellow (n=3)	
	Clarity	Clear (n=3)	Cloudy (n=3)		Sl cloudy (n=1) Clear (n=2)	Sl cloudy (n=2) Cloudy (n=1)	
	Nitrates[A]	Negative (n=3)	Negative (n=3)		Negative (n=3)	Negative (n=3)	
	Glucose[A]	Negative (n=3)	Negative (n=3)		Negative (n=3)	Negative (n=3)	
	Ketones[A]	Negative (n=3)	1+ (n=1) Negative (n=2)		Negative (n=3)	Trace (n=1) 1+ (n=1) 2+ (n=1)	
	Blood[A]	Negative (n=3)	Trace (n=2) Negative (n=1)		Trace (n=1) Negative (n=2)	Trace (n=1) 1+ (n=1) 3+ (n=1)	
	Protein[A]	Negative (n=3)	Trace (n=1) Negative (n=2)		Negative (n=3)	1+ (n=1) Negative (n=2)	
	Bilirubin[A]	Negative (n=3)	Negative (n=3)		Negative (n=3)	Negative (n=3)	
	pH[A]	8.0 (n=1) 8.5 (n=2)	8.0 (n=1) 8.5 (n=1) >=9.0 (n=1)		8.0 (n=1) 8.5 (n=2)	7.5 (n=2) 8.5 (n=1)	
	Leukocytes[A]	Negative (n=3)	Negative (n=3)		Trace (n=1) 1+ (n=1) Negative (n=1)	Trace (n=1) 2+ (n=1) Negative (n=1)	
	Urobilirubin (EU/dL)	0.2 (n=3)	0.2 (n=3)		0.2 (n=3)	0.2 (n=3)	

Table S15. continued.

Dose group (mg/kg/day)	ECG parameter	Male			Female		
		Day -6	Day 15	Day 41 (recovery animals)	Day -6	Day 15	Day 41 (recovery animals)
50 (n=3)	Specific gravity	1.024 ± 0.005	1.027 ± 0.012		1.013 ± 0.009	1.015 ± 0.006	
	Volume (mL)	50.0 ± 26.5	65.0 ± 86.6		90.0 ± 26.5	18.0 ± 5.3	
	Creatinine (mg/dL)	93.0 ± 21.1	115.2 ± 45.7		40.4 ± 26.8	73.7 ± 12.6	
	Sodium (mEq/L)	49.2 ± 26.3	17.0 ± 6.8		19.5 ± 10.4	23.3 ± 4.9	
	Potassium (mEq/L)	92.3 ± 25.5	45.7 ± 28.6		72.6 ± 46.8	39.0 ± 16.7	
	Chloride (mEq/L)	47.1 ± 10.5	50.2 ± 3.5		32.5 ± 15.0	43.2 ± 19.6	
	Colour	Yellow (n=3)	Yellow (n=3)		Yellow (n=3)	Brown (n=1) Yellow (n=2)	
	Clarity	Clear (n=3)	Sl cloudy (n=1) Clear (n=2)		Cloudy (n=1) Clear (n=2)	Cloudy (n=1) Sl cloudy (n=2)	
	Nitrates[A]	Negative (n=3)	Negative (n=3)		Negative (n=3)	Negative (n=3)	
	Glucose[A]	Negative (n=3)	Negative (n=3)		Negative (n=3)	Trace (n=1) Negative (n=2)	
	Ketones[A]	Negative (n=3)	2+ (n=2) Negative (n=1)		Negative (n=3)	Trace (n=1) 1+ (n=1) 2+ (n=1)	
	Blood[A]	Negative (n=3)	Trace (n=1) Negative (n=2)		Trace (n=1) Negative (n=2)	Trace (n=2) 3+ (n=1)	
	Protein[A]	Negative (n=3)	Trace (n=1) Negative (n=2)		Negative (n=3)	2+ (n=1) Negative (n=2)	
	Bilirubin[A]	Negative (n=3)	Negative (n=3)		Negative (n=3)	Negative (n=3)	
	pH[A]	8.0 (n=1) 8.5 (n=2)	6.0 (n=1) 7.0 (n=2)		7.5 (n=1) 8.0 (n=1) 8.5 (n=1)	7.0 (n=2) 8.0 (n=1)	
	Leukocytes[A]	Negative (n=3)	Negative (n=3)		Trace (n=2) Negative (n=1)	2+ (n=2) Negative (n=1)	
	Urobilirubin (EU/dL)	0.2 (n=3)	0.2 (n=3)		0.2 (n=3)	0.2 (n=2) 4.0 (n=1)	
100 (n=5)	Specific gravity	1.021 ± 0.006	1.027 ± 0.012	1.016 ± 0.004	1.024 ± 0.006	1.020 ± 0.009	1.019 ± 0.008
	Volume (mL)	64.0 ± 25.1	56.0 ± 54.9	45.0 ± 24.0	78.0 ± 29.5	42.0 ± 25.8	100.5 ± 17.7
	Creatinine (mg/dL)	81.1 ± 17.6	115.2 ± 45.7	63.9 ± 8.4	75.8 ± 21.4	89.7 ± 47.1	52.1 ± 20.8
	Sodium (mEq/L)	37.4 ± 18.5	39.4 ± 36.0	29.7 ± 1.4	34.9 ± 13.5	20.0 ± 7.2	40.5 ± 24.4
	Potassium (mEq/L)	92.6 ± 30.9	52.4 ± 20.7	64.9 ± 4.9	112.6 ± 27.5	49.2 ± 9.3	99.0 ± 51.8
	Chloride (mEq/L)	44.4 ± 11.7	53.4 ± 13.9	55.5 ± 8.9	66.1 ± 20.0	55.1 ± 21.1	64.5 ± 27.6
	Colour	Yellow (n=5)	Yellow (n=5)	Yellow (n=2)	Brown (n=1) Yellow (n=4)	Yellow (n=5)	Yellow (n=2)
	Clarity	Clear (n=5)	Cloudy (n=2) Sl cloudy n=2)	Cloudy (n=1) Sl cloudy (n=1)	Sl cloudy (n=2) Clear (n=3)	Sl cloudy (n=4) Clear (n=1)	Sl cloudy (n=1) Cloudy (n=1)
	Nitrates[A]	Negative (n=5)	Negative (n=5)	Negative (n=2)	Positive (n=1) Negative (n=4)	Negative (n=5)	Positive (n=1) Negative (n=1)
	Glucose[A]	Negative (n=5)	Negative (n=5)	Negative (n=2)	Negative (n=5)	Negative (n=5)	Negative (n=2)
	Ketones[A]	2+ (n=1) Negative (n=4)	Trace (n=1) 2+ (n=1) 3+ (n=1) Negative (n=2)	Negative (n=2)	Negative (n=5)	Trace (n=1) 2+ (n=1) 3+ (n=1) Negative (n=2)	Negative (n=2)
	Blood[A]	Negative (n=5)	3+ (n=1) Negative (n=4)	Trace (n=1) Negative (n=1)	1+ (n=1) 2+ (n=1) 3+ (n=1) Negative (n=2)	1+ (n=3) 2+ (n=1) 3+ (n=1)	Negative (n=2)
	Protein[A]	Negative (n=5)	Trace (n=3) Negative (n=2)	Negative (n=2)	Negative (n=5)	Trace (n=2) Negative (n=3)	Negative (n=2)
	Bilirubin[A]	Negative (n=5)	Negative (n=5)	Negative (n=2)	Negative (n=3)	Negative (n=3)	Negative (n=2)
	pH[A]	8.5 (n=5)	6.0 (n=1) 7.5 (n=2) 8.0 (n=1) 8.5 (n=1)	7.0 (n=1) 8.5 (n=1)	8.0 (n=1) 8.5 (n=4)	6.0 (n=1) 7.0 (n=1) 7.5 (n=1) 8.5 (n=2)	8.5 (n=2)
	Leukocytes[A]	Negative (n=5)	Negative (n=5)	Negative (n=2)	Trace (n=3) 1+ (n=1) Negative (n=1)	Trace (n=2) 2+ (n=1) Negative (n=2)	Negative (n=2)
	Urobilirubin (EU/dL)	0.2 (n=5)	0.2 (n=4) 1.0 (n=1)	0.2 (n=2)	0.2 (n=5)	0.2 (n=5)	0.2 (n=2)

Data are presented as mean ± SD.

[A] Urine strips are read with a qualitative result (negative to 4+) to reflect the nature of the test.

Abbreviations: n (number of animals); Sl (slightly).

Table S16. Blood gas parameters in cynomolgus monkeys.

Day	Parameter	Male				Female			
		Vehicle	25 mg/kg/ day	50 mg/kg/ day	100 mg/kg/ day	Vehicle	25 mg/kg/ day	50 mg/kg/ day	100 mg/kg/ day
Day -2	No. of animals	n=5	n=3	n=3	n=5	n=5	n=3	n=3	n=5
	pH	7.46 ± 0.09	7.50 ± 0.05	7.53 ± 0.03	7.49 ± 0.07	7.50 ± 0.04	7.50 ± 0.01	7.48 ± 0.1	7.50 ± 0.02
	PCO2 (mmHg)	32.1 ± 2.8	34.6 ± 3.3	32.0 ± 1.3	30.5 ± 1.6	32.3 ± 2.8	36.2 ± 0.4	32.2 ± 1.0	31.2 ± 2.9
	PO2 (mmHg)	87 ± 6	80 ± 9	72 ± 10	89 ± 5	87 ± 4	84 ± 11	86 ± 10	91 ± 4
	BEecf (mmol/L)	-1 ± 7	4 ± 6	4 ± 1	0 ± 6	2 ± 4	5 ± 1	1 ± 5	1 ± 2
	HCO_3 (mmol/L)	23.3 ± 5.8	27.0 ± 4.9	26.7 ± 0.8	23.4 ± 4.5	25.2 ± 3.9	28.2 ± 0.9	24.1 ± 4.1	24.3 ± 1.9
	TCO_2 (mmol/L)	24 ± 6	28 ± 5	28 ± 1	24 ± 5	26 ± 4	29 ± 1	25 ± 4	25 ± 2
	sO_2 (%)	97 ± 1	97 ± 1	96 ± 2	98 ± 1	98 ± 1	97 ± 1	97 ± 1	98 ± 0
	Lactate (mmol/L)	5.13 ± 6.39	4.06 ± 5.34	3.10 ± 1.66	5.61 ± 4.20	3.84 ± 2.64	1.61 ± 0.9	5.79 ± 4.28	3.24 ± 1.09
Day 14	No. of animals	n=5	n=3	n=3	n=5	n=5	n=3	n=3	n=5
	pH	7.43 ± 0.06	7.47 ± 0.04	7.39 ± 0.1	7.45 ± 0.10	7.47 ± 0.03	7.47 ± 0.02	7.52 ± 0.03	7.42 ± 0.08
	PCO2 (mmHg)	31.8 ± 2.5	31.3 ± 3.2	30.4 ± 1.2	32.5 ± 3.3	31.3 ± 2.1	33.3 ± 2.2	30.3 ± 1.3	32.0 ± 2.7
	PO2 (mmHg)	88 ± 6	94 ± 6	87 ± 3	88 ± 6	90 ± 9	81 ± 7	94 ± 7	94 ± 7
	BEecf (mmol/L)	-3 ± 4	-1 ± 5	-6 ± 5	-1 ± 8	-1 ± 3	0 ± 2	2 ± 2	-3 ± 7
	HCO_3 (mmol/L)	21.5 ± 3.5	22.7 ± 4.2	18.7 ± 3.7	23.3 ± 6.0	23.0 ± 2.9	24.0 ± 1.4	24.7 ± 1.3	21.3 ± 5.4
	TCO_2 (mmol/L)	23 ± 4	24 ± 4	19 ± 4	24 ± 6	24 ± 3	25 ± 2	25 ± 1	22 ± 6
	sO_2 (%)	97 ± 0	98 ± 0	97 ± 1	97 ± 1	97 ± 1	97 ± 2	98 ± 1	98 ± 0
	Lactate (mmol/L)	5.05 ± 3.87	4.81 ± 2.95	7.40 ± 6.02	7.05 ± 5.69	3.64 ± 1.78	4.08 ± 1.56	2.25 ± 0.53	5.71 ± 4.28

Data are presented as mean ± SD.
Abbreviations: BEecf (Base Excess); No (number); sO_2 (oxygen saturation).

Table S17A. Immunophenotyping (relative counts) in cynomolgus monkeys.

Day	Cell type	Male				Female			
		Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day	Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day
Acclimation	No. of animals	n=5	n=3	n=3	n=5	n=5	n=3	n=3	n=5
	Total T-lymphocytes $CD3^+$ (%)	58.4 ± 5.0	61.8 ± 13.8	51.7 ± 2.3	62.8 ± 13.1	61.1 ± 6.6	53.8 ± 12.8	64.6 ± 11.7	58.6 ± 3.6
	T-helper $CD3^+$ $CD4^+$ (%)	36.3 ± 4.3	46.4 ± 11.3	30.5 ± 6.3	33.5 ± 10.2	36.4 ± 8.5	33.6 ± 10.8	39.7 ± 8.7	40.1 ± 2.8
	T-cytotoxic $CD3^+$ $CD8a^+$ (%)	17.0 ± 3.1	11.9 ± 2.0	12.2 ± 2.7	25.8 ± 5.0	17.5 ± 2.1	14.6 ± 2.3	18.2 ± 5.5	15.9 ± 3.3
	Reg T Cells $CD25^+$ (%)	4.6 ± 1.1	6.2 ± 1.7	7.4 ± 5.2	6.7 ± 2.3	4.5 ± 1.4	5.1 ± 2.4	6.1 ± 5.3	5.0 ± 2.1
	NK cells $CD3^+$ $CD16^+$ (%)	11.5 ± 5.1	10.1 ± 1.4	16.8 ± 1.9	11.9 ± 7.1	11.2 ± 6.3	9.8 ± 3.1	9.5 ± 4.9	12.1 ± 8.0
	B cells $CD20^+$ cells (%)	22.5 ± 5.3	21.0 ± 11.2	24.5 ± 2.0	22.2 ± 14.4	24.0 ± 6.0	32.6 ± 8.4	21.7 ± 6.4	24.7 ± 8.5
	Activated T cells $CD69^+$ (%)	1.7 ± 0.9	3.3 ± 1.8	3.6 ± 1.2	1.7 ± 1.3	3.5 ± 3.2	3.1 ± 2.4	6.1 ± 7.7	2.5 ± 1.4
	Activated NK cells $CD69^+$ (%)	39.0 ± 23.4	42.6 ± 8.7	38.2 ± 25.4	26.5 ± 20.1	28.5 ± 14.5	43.0 ± 24.4	39.6 ± 7.9	34.7 ± 6.0
	Activated B cells $CD69^+$ (%)	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.6 ± 0.3	0.3 ± 0.1
Day 15	No. of animals	n=5	n=3	n=3	n=5	n=5	n=3	n=3	n=5
	Total T-lymphocytes $CD3^+$ (%)	59.8 ± 3.7	60.3 ± 13.9	55.8 ± 4.0	63.6 ± 12.5	57.6 ± 7.5	52.9 ± 12.4	66.7 ± 7.7	61.7 ± 2.3
	T-helper $CD3^+$ $CD4^+$ (%)	37.4 ± 3.1	42.7 ± 12.4	32.5 ± 2.9	34.6 ± 8.3	36.4 ± 8.7	34.8 ± 10.1	44.7 ± 3.9	43.1 ± 2.4
	T-cytotoxic $CD3^+$ $CD8a^+$ (%)	18.7 ± 3.9	12.7 ± 0.8	17.1 ± 2.7	25.4 ± 4.7	16.6 ± 2.2	16.9 ± 3.5	19.9 ± 2.5	17.2 ± 4.0
	Reg T Cells $CD25^+$ (%)	5.2 ± 1.1	6.8 ± 1.9	8.4 ± 6.4	7.2 ± 2.6	5.2 ± 0.7	7.0 ± 3.2	6.3 ± 4.8	5.8 ± 3.4
	NK cells $CD3^+$ $CD16^+$ (%)	8.5 ± 3.9	12.0 ± 3.0	14.3 ± 4.0	8.9 ± 3.2	7.5 ± 2.3	11.1 ± 3.0	5.6 ± 4.4	10.5 ± 5.4
	B cells $CD20^+$ cells (%)	26.3 ± 2.3	23.0 ± 9.2	23.9 ± 1.3	23.0 ± 10.6	28.5 ± 10.0	30.1 ± 10.2	22.8 ± 2.2	23.6 ± 6.7
	Activated T cells $CD69^+$ (%)	1.3 ± 0.4	4.2 ± 3.0	3.2 ± 1.6	1.4 ± 0.8	2.2 ± 2.2	2.6 ± 1.1	2.1 ± 1.8	1.9 ± 0.8
	Activated NK cells $CD69^+$ (%)	37.7 ± 22.6	44.7 ± 6.1	38.4 ± 16.0	27.4 ± 17.8	24.8 ± 11.5	38.0 ± 18.5	37.9 ± 22.0	31.3 ± 9.6
	Activated B cells $CD69^+$ (%)	0.5 ± 0.2	0.7 ± 0.5	0.5 ± 0.2	0.5 ± 0.2	0.4 ± 0.1	0.7 ± 0.4	0.6 ± 0.1	0.5 ± 0.2
Day 41 (recovery animals)	No. of animals	n=2	n=0	n=0	n=2	n=2	n=0	n=0	n=2
	Total T-lymphocytes $CD3^+$ (%)	68.8	n.d.	n.d.	26.1	64.6	;n.d.	n.d.	65.2
	T-helper $CD3^+$ $CD4^+$ (%)	32.5	n.d.	n.d.	31.1	36.7	n.d.	n.d.	44.2
	T-cytotoxic $CD3^+$ $CD8a^+$ (%)	24.0	n.d.	n.d.	9.0	21.5	n.d.	n.d.	15.2
	Reg T Cells $CD25^+$ (%)	4.8	n.d.	n.d.	7.2	4.5	n.d.	n.d.	4.6
	NK cells $CD3^+$ $CD16^+$ (%)	8.9	n.d.	n.d.	7.8	7.1	n.d.	n.d.	11.6
	B cells $CD20^+$ cells (%)	18.4	n.d.	n.d.	27.5	24.8	n.d.	n.d.	19.9
	Activated T cells $CD69^+$ (%)	3.9	n.d.	n.d.	3.7	3.1	n.d.	n.d.	3.8
	Activated NK cells $CD69^+$ (%)	46.6	n.d.	n.d.	20.3	31.5	n.d.	n.d.	47.6
	Activated B cells $CD69^+$ (%)	0.4	n.d.	n.d.	0.3	0.4	n.d.	n.d.	0.6

Data are presented as mean ± SD.
Abbreviations: n.d. (not determined); n (number of animals).

Table S17B. Immunophenotyping (absolute counts) in cynomolgus monkeys.

Day	Cell type	Male				Female			
		Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day	Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day
Acclimation	No. of animals	n=5	n=3	n=3	n=5	n=5	n=3	n=3	n=5
	Total T-lymphocytes $CD3^+$ ($10^3/\mu L$)	3.48 ± 0.81	2.09 ± 0.58	2.09 ± 0.95	3.18 ± 1.50	2.85 ± 1.18	2.03 ± 0.77	2.84 ± 1.67	2.50 ± 0.93
	T-helper $CD3^+$ $CD4^+$ ($10^3/\mu L$)	2.22 ± 0.72	1.57 ± 0.46	1.17 ± 0.45	1.62 ± 0.68	1.64 ± 0.62	1.26 ± 0.47	1.84 ± 1.35	1.70 ± 0.64
	T-cytotoxic $CD3^+$ $CD8^+$ ($10^3/\mu L$)	0.99 ± 0.19	0.44 ± 0.24	0.46 ± 0.13	1.30 ± 0.62	0.83 ± 0.38	0.58 ± 0.27	0.78 ± 0.44	0.66 ± 0.23
	Reg T Cells $CD25^+$ ($10^3/\mu L$)	0.10 ± 0.04	0.10 ± 0.04	0.07 ± 0.02	0.11 ± 0.06	0.07 ± 0.02	0.06 ± 0.01	0.10 ± 0.07	0.09 ± 0.05
	NK cells $CD3^+CD16^+$ ($10^3/\mu L$)	0.69 ± 0.35	0.37 ± 0.18	0.69 ± 0.34	0.49 ± 0.11	0.48 ± 0.20	0.37 ± 0.16	0.38 ± 0.17	0.43 ± 0.17
	B cells $CD20^+$ cells ($10^3/\mu L$)	1.37 ± 0.46	0.80 ± 0.56	0.97 ± 0.42	1.46 ± 1.87	1.22 ± 0.77	1.32 ± 0.66	0.92 ± 0.41	1.15 ± 0.73
	Activated T cells $CD69^+$ ($10^3/\mu L$)	0.06 ± 0.04	0.06 ± 0.02	0.08 ± 0.05	0.05 ± 0.04	0.11 ± 0.13	0.06 ± 0.04	0.13 ± 0.15	0.06 ± 0.04
	Activated NK cells $CD69^+$ ($10^3/\mu L$)	0.33 ± 0.34	0.15 ± 0.07	0.28 ± 0.26	0.12 ± 0.09	0.14 ± 0.08	0.13 ± 0.02	0.16 ± 0.09	0.15 ± 0.07
	Activated B cells $CD69^+$ ($10^3/\mu L$)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
Day 15	No. of animals	n=5	n=3	n=3	n=5	n=5	n=3	n=3	n=5
	Total T-lymphocytes $CD3^+$ ($10^3/\mu L$)	3.37 ± 0.90	2.62 ± 0.41	2.24 ± 0.87	3.00 ± 0.45	2.35 ± 0.54	2.05 ± 0.89	2.38 ± 1.22	2.36 ± 0.95
	T-helper $CD3^+$ $CD4^+$ ($10^3/\mu L$)	2.13 ± 0.69	1.85 ± 0.38	1.30 ± 0.51	1.62 ± 0.26	1.46 ± 0.33	1.34 ± 0.55	1.59 ± 0.82	1.64 ± 0.66
	T-cytotoxic $CD3^+$ $CD8^+$ ($10^3/\mu L$)	1.05 ± 0.30	0.56 ± 0.07	0.66 ± 0.18	1.23 ± 0.32	0.70 ± 0.25	0.67 ± 0.33	0.69 ± 0.30	0.65 ± 0.25
	Reg T Cells $CD25^+$ ($10^3/\mu L$)	0.11 ± 0.03	0.12 ± 0.02	0.09 ± 0.04	0.11 ± 0.04	0.08 ± 0.03	0.08 ± 0.00	0.09 ± 0.06	0.10 ± 0.08
	NK cells $CD3^+CD16^+$ ($10^3/\mu L$)	0.45 ± 0.18	0.54 ± 0.17	0.53 ± 0.03	0.42 ± 0.12	0.30 ± 0.07	0.40 ± 0.06	0.15 ± 0.09	0.34 ± 0.06
	B cells $CD20^+$ cells ($10^3/\mu L$)	1.47 ± 0.36	1.03 ± 0.46	0.95 ± 0.35	1.20 ± 0.86	1.30 ± 0.87	1.17 ± 0.52	0.80 ± 0.39	0.98 ± 0.58
	Activated T cells $CD69^+$ ($10^3/\mu L$)	0.04 ± 0.02	0.12 ± 0.10	0.07 ± 0.03	0.04 ± 0.02	0.06 ± 0.06	0.05 ± 0.03	0.05 ± 0.04	0.04 ± 0.03
	Activated NK cells $CD69^+$ ($10^3/\mu L$)	0.19 ± 0.15	0.24 ± 0.09	0.20 ± 0.09	0.12 ± 0.09	0.08 ± 0.04	0.15 ± 0.05	0.05 ± 0.03	0.11 ± 0.03
	Activated B cells $CD69^+$ ($10^3/\mu L$)	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Day 41 (recovery animals)	No. of animals	n=2	n=0	n=0	n=2	n=2	n=0	n=0	n=2
	Total T-lymphocytes $CD3^+$ ($10^3/\mu L$)	6.55	n.d.	n.d.	5.41	5.56	;n.d.	n.d.	4.48
	T-helper $CD3^+$ $CD4^+$ ($10^3/\mu L$)	3.06	n.d.	n.d.	2.19	3.16	n.d.	n.d.	3.10
	T-cytotoxic $CD3^+$ $CD8^+$ ($10^3/\mu L$)	2.29	n.d.	n.d.	2.89	1.86	n.d.	n.d.	1.03
	Reg T Cells $CD25^+$ ($10^3/\mu L$)	0.15	n.d.	n.d.	0.20	0.14	n.d.	n.d.	0.15
	NK cells $CD3^+CD16^+$ ($10^3/\mu L$)	0.81	n.d.	n.d.	0.60	0.60	n.d.	n.d.	0.62
	B cells $CD20^+$ cells ($10^3/\mu L$)	1.72	n.d.	n.d.	3.38	2.53	n.d.	n.d.	1.51
	Activated T cells $CD69^+$ ($10^3/\mu L$)	0.26	n.d.	n.d.	0.21	0.18	n.d.	n.d.	0.14
	Activated NK cells $CD69^+$ ($10^3/\mu L$)	0.40	n.d.	n.d.	0.13	0.20	n.d.	n.d.	0.30
	Activated B cells $CD69^+$ ($10^3/\mu L$)	0.01	n.d.	n.d.	0.00	0.01	n.d.	n.d.	0.01

Data are presented as mean ± SD.
Abbreviations: n.d. (not determined); n (number of animals).

Table S18. Cytokines in cynomolgus monkeys.

Day	Cytokines	Male & female
		100 mg/kg/day
Pre treatment	No. of animals	n=10
	IL-1β (pg/mL)	16.92 ± 2.74
	IFN-γ (pg/mL)	151.41 ± 271.20
	TNF-α (pg/mL)	ND
	IL-6 (pg/mL)	66.55 ± ND
	IL-8 (pg/mL)	263.46 ± 225.12
	IL-4 (pg/mL)	ND
	IL-12 (pg/mL)	624.88 ± 174.44
	IL-2 (pg/mL)	219.95 ± 56.50
	IL-10 (pg/mL)	ND
8 hours	No. of animals	n=10
	IL-1β (pg/mL)	18.60 ± 3.21
	IFN-γ (pg/mL)	113.37 ± 186.99
	TNF-α (pg/mL)	ND
	IL-6 (pg/mL)	28.93 ± 6.85
	IL-8 (pg/mL)	450.00 ± 370.62
	IL-4 (pg/mL)	ND
	IL-12 (pg/mL)	478.52 ± 71.65
	IL-2 (pg/mL)	539.45 ± 507.52
	IL-10 (pg/mL)	ND

Data are presented as mean ± SD. All results below the quantification limit or value could not be determined.
Abbreviations: IFN-γ (interferon gamma); IL (Interleukin); ND (no data); TNF-α (Tumor Necrosis Factor alpha).

Table S19. Plasma IgE levels in cynomolgus monkeys.

		Male			
		Vehicle (n=5)	25 mg/kg/day (n=3)	50 mg/kg/day (n=3)	100 mg/kg/day (n=5)
Day -6	IgE	1028 [566.6-64657]	1180 [456.5-12229]	3090 [1253-6037]	868.3 [425.3-1550]
Day 15	IgE	1668 [575.9-80837]	2144 [1598-61648]	3929 [1230-5382]	1117 [845.5-2748]
		Female			
Day -6	IgE	2533 [517.9-5488]	782.7 [596.7-987.8]	1587 [227.4-10925]	1639 [424.3-5087]
Day 15	IgE	5087 [1316-80685]	885.8 [703.9-944.1]	1448 [137.5-7526]	2697 [460.4-5122]

Data are presented as median [interquartile range].
Abbreviations: IgE (immuneglobulin E); n (number of animals).

Table S20. Maccroscopic abnormalities in cynomolgus monkeys.

	14 day follow-up								Recovery animals			
	Vehicle		25 mg/kg/day		50 mg/kg/day		100 mg/kg/day		Vehicle		100 mg/kg/day	
Gender	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Adrenal glands	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Aorta	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Bone marrow, sternum	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Bone, femur	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Bone, sternum	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Brain	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Cervix	-	N (n=3)	-	N (n=3)	-	N (n=3)	-	N (n=3)	-	N (n=2)	-	N (n=2)
Epididymis	N (n=3)	-	N (n=3)	-	N (n=3)	-	N (n=3)	-	N (n=2)	-	N (n=2)	-
Esophagus	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Eyes	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Gallbladder	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Heart	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Injection site(s)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Intestine, cecum	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Intestine, colon	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Intestine, ileum	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Intestine, jejunum	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Intestine, rectum	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Kidneys	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Liver	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Lungs	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Lymph node, mesenteric	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Lymph nodes, mandibular	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Mammary gland	-	N (n=3)	-	N (n=3)	-	N (n=3)	-	N (n=3)	-	N (n=2)	-	N (n=2)
Nerves, optic	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Nerve, sciatic	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	-	N (n=2)	-	N (n=2)
Ovaries	-	N (n=3)	-	N (n=3)	-	N (n=3)	-	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Pancreas	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Parathyroid gland	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)

Table S20. continued.

	14 day follow-up								Recovery animals			
	Vehicle		25 mg/kg/day		50 mg/kg/day		100 mg/kg/day		Vehicle		100 mg/kg/day	
Gender	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Pituitary gland	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Prostate gland	N (n=3)	-	N (n=3)	-	N (n=3)	-	N (n=3)	-	N (n=2)	-	N (n=2)	-
Salivary glands, submandibular	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Seminal vesicles	N (n=3)	-	N (n=3)	-	N (n=3)	-	N (n=3)	-	N (n=2)	-	N (n=2)	-
Skeletal muscle	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Skin	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Spinal cord, thoracic	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Spleen	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Stomach	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Testes	N (n=3)	-	N (n=3)	-	N (n=3)	-	N (n=3)	-	N (n=2)	-	N (n=2)	-
Thymus	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Thyroid gland	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Tongue	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Trachea	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=2)	N (n=2)	N (n=2)
Urinary bladder	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=3)	N (n=2)	N (n=1)	N (n=2)	N (n=2)
Dark red mucosa	-	-	-	-	-	-	-	-	-	n=1	-	-
Uterus	-	N (n=3)	-	N (n=3)	-	N (n=3)	-	N (n=3)	-	N (n=2)	-	N (n=2)
Vagina	-	N (n=3)	-	N (n=3)	-	N (n=3)	-	N (n=3)	-	N (n=2)	-	N (n=2)

Abbreviations: N (normal); n (number of animals).

Table S21. Organ weights in cynomolgus monkeys.

Gender	Organ		14 day follow-up				42-day follow-up	
			Vehicle (n=3)	25 mg/kg/day (n=3)	50 mg/kg/day (n=3)	100 mg/kg/day (n=3)	Vehicle (n=2)	100 mg/kg/day (n=2)
Male	Total body weight	Absolute (kg)	2.417 ± 0.268	2.934 ± 0.966	2.552 ± 0.208	3.210 ± 0.827	2.733 ± 0.325	2.805 ± 0.330
	Adrenals	Absolute (gram)	0.438 ± 0.085	0.582 ± 0.109	0.492 ± 0.062	0.504 ± 0.124	0.363 ± 0.107	0.342 ± 0.013
		Relative to BW (%)	0.018 ± 0.002	0.022 ± 0.010	0.019 ± 0.003	0.016 ± 0.002	0.014 ± 0.002	0.012 ± 0.001
	Brain	Absolute (gram)	75.132 ± 4.515	68.101 ± 1.794	71.174 ± 1.570	66.296 ± 2.645	69.761 ± 4.363	69.929 ± 1.942
		Relative to BW (%)	3.129 ± 0.319	2.490 ± 0.787	2.799 ± 0.183	2.173 ± 0.627	2.562 ± 0.145	2.495 ± 0.224
	Epididymides	Absolute (gram)	0.563 ± 0.167	1.052 ± 0.471	0.867 ± 0.674	2.075 ± 1.956	0.593 ± 0.332	0.909 ± 0.792
		Relative to BW (%)	0.749 ± 0.220	0.035 ± 0.004	0.033 ± 0.024	0.058 ± 0.044	0.021 ± 0.010	0.031 ± 0.025
	Heart	Absolute (gram)	7.905 ± 1.185	10.328 ± 3.008	8.746 ± 0.731	11.425 ± 2.328	10.454 ± 4.002	10.165 ± 0.895
		Relative to BW (%)	0.327 ± 0.027	0.355 ± 0.043	0.343 ± 0.017	0.361 ± 0.048	0.377 ± 0.102	0.367 ± 0.075
	Kidneys	Absolute (gram)	10.215 ± 1.298	11.958 ± 2.154	10.116 ± 0.496	13.821 ± 2.214	11.480 ± 3.427	10.593 ± 0.738
		Relative to BW (%)	0.424 ± 0.052	0.421 ± 0.065	0.397 ± 0.016	0.441 ± 0.068	0.416 ± 0.076	0.379 ± 0.018
	Liver w/ gallbladder	Absolute (gram)	48.150 ± 8.197	57.176 ± 15.248	47.695 ± 4.348	60.412 ± 10.068	57.565± 11.244	58.106 ± 1.029
		Relative to BW (%)	1.983 ± 0.133	1.973 ± 0.115	1.873 ± 0.160	1.917 ± 0.228	2.097 ± 0.162	2.084 ± 0.208
	Pituitary	Absolute (gram)	0.049 ± 0.005	0.043 ± 0.009	0.042 ± 0.019	0.055 ± 0.008	0.044 ± 0.010	0.045 ± 0.002
		Relative to BW (%)	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.001	0.002 ± 0.001	0.002 ± 0.000	0.002 ± 0.000
	Prostate w/ seminal v	Absolute (gram)	0.853 ± 0.290	2.670 ± 2.807	1.093 ± 0.558	3.862 ± 4.011	0.816 ± 0.443	1.273 ± 0.711
		Relative to BW (%)	0.035 ± 0.009	0.078 ± 0.061	0.042 ± 0.019	0.106 ± 0.092	0.029 ± 0.013	0.044 ± 0.020
	Spleen	Absolute (gram)	3.056 ± 1.070	3.801 ± 1.978	4.410 ± 1.052	3.725 ± 1.171	3.777 ± 0.353	3.920 ± 1.013
		Relative to BW (%)	0.125 ± 0.033	0.125 ± 0.023	0.171 ± 0.029	0.117 ± 0.029	0.138 ± 0.004	0.143 ± 0.053
	Testes	Absolute (gram)	0.826 ± 0.285	1.951 ± 1.693	0.990 ± 0.490	10.678 ± 15.830	1.086 ± 0.931	1.398 ± 0.165
		Relative to BW (%)	0.034 ± 0.009	0.060 ± 0.033	0.038 ± 0.016	0.276 ± 0.389	0.038 ± 0.030	0.050 ± 0.000
	Thymus	Absolute (gram)	3.347 ± 1.978	2.180 ± 1.092	2.120 ± 0.881	2.616 ± 1.586	4.340 ± 3.301	3.128 ± 0.853
		Relative to BW (%)	0.134 ± 0.070	0.079 ± 0.050	0.083 ± 0.031	0.086 ± 0.050	0.153 ± 0.103	0.111 ± 0.017
	Thyroids w/ parathyroids	Absolute (gram)	0.302 ± 0.157	0.462 ± 0.101	0.316 ± 0.070	0.435 ± 0.194	0.410 ± 0.227	0.533 ± 0.249
		Relative to BW (%)	0.012 ± 0.006	0.016 ± 0.004	0.013 ± 0.004	0.014 ± 0.004	0.015 ± 0.007	0.019 ± 0.007

Table S21. continued

Gender	Organ		14 day follow-up				42-day follow-up	
			Vehicle (n=3)	25 mg/kg/day (n=3)	50 mg/kg/day (n=3)	100 mg/kg/day (n=3)	Vehicle (n=2)	100 mg/kg/day (n=2)
Female	Total body weight	Absolute (kg)	2.786 ± 0.633	2.956 ± 0.686	2.352 ± 0.137	2.727 ± 0.472	2.907 ± 0.028	2.546 ± 0.086
	Adrenals	Absolute (gram)	0.549 ± 0.089	0.569 ± 0.186	0.464 ± 0.140	0.548 ± 0.097	0.448 ± 0.190	0.357 ± 0.062
		Relative to BW (%)	0.020 ± 0.002	0.019 ± 0.002	0.020 ± 0.006	0.020 ± 0.000	0.015 ± 0.006	0.014 ± 0.003
	Brain	Absolute (gram)	61.080 ± 1.20	60.331 ± 8.125	61.632 ± 2.304	69.734 ± 12.941	61.096 ± 7.148	63.596 ± 5.444
		Relative to BW (%)	2.263 ± 0.470	2.072 ± 0.225	2.623 ± 0.085	2.598 ± 0.567	2.101 ± 0.225	2.496 ± 0.129
	Heart	Absolute (gram)	8.970 ± 1.497	10.589 ± 1.366	8.190 ± 0.290	9.433 ± 2.087	10.032 ± 0.231	9.147 ± 1.010
		Relative to BW (%)	0.326 ± 0.038	0.364 ± 0.035	0.349 ± 0.017	0.344 ± 0.017	0.345 ± 0.005	0.359 ± 0.027
	Kidneys	Absolute (gram)	10.893 ± 0.680	11.154 ± 0.490	9.540 ± 1.613	12.016 ± 2.016	11.529 ± 1.493	10.668 ± 0.234
		Relative to BW (%)	0.401 ± 0.066	0.389 ± 0.076	0.404 ± 0.045	0.441 ± 0.007	0.397 ± 0.055	0.419 ± 0.023
	Liver w/ gallbladder	Absolute (gram)	56.733 ± 10.229	61.660 ± 9.989	54.650 ± 11.291	54.222 ± 10.333	58.133 ± 5.579	50.966 ± 3.892
		Relative to BW (%)	2.056 ± 0.201	2.126 ± 0.393	2.322 ± 0.453	1.984 ± 0.060	1.999 ± 0.172	2.000 ± 0.085
	Ovaries	Absolute (gram)	0.368 ± 0.017	0.248 ± 0.393	0.235 ± 0.042	0.274 ± 0.105	0.288 ± 0.059	0.360 ± 0.086
		Relative to BW (%)	0.014 ± 0.003	0.008 ± 0.001	0.010 ± 0.002	0.010 ± 0.004	0.010 ± 0.002	0.014 ± 0.004
	Pituitary	Absolute (gram)	0.055 ± 0.019	0.061 ± 0.020	0.032 ± 0.003	0.053 ± 0.021	0.065 ± 0.025	0.047 ± 0.001
		Relative to BW (%)	0.002 ± 0.000	0.002 ± 0.000	0.001 ± 0.000	0.002 ± 0.001	0.002 ± 0.001	0.002 ± 0.000
	Spleen	Absolute (gram)	2.894 ± 0.524	3.506 ± 0.693	5.024 ± 2.440	4.101 ± 0.466	4.747 ± 1.594	3.000 ± 0.916
		Relative to BW (%)	0.105 ± 0.005	0.120 ± 0.023	0.212 ± 0.010	0.151 ± 0.011	0.164 ± 0.056	0.119 ± 0.040
	Thymus	Absolute (gram)	1.475 ± 0.652	2.546 ± 0.732	2.064 ± 0.718	1.728 ± 0.459	3.611 ± 0.714	1.758 ± 0.221
		Relative to BW (%)	0.056 ± 0.028	0.406 ± 0.296	0.088 ± 0.029	0.066 ± 0.024	0.124 ± 0.023	0.069 ± 0.011
	Thyroids w/ parathyroids	Absolute (gram)	0.324 ± 0.057	0.407 ± 0.296	0.452 ± 0.046	0.360 ± 0.099	0.418 ± 0.051	0.346 ± 0.030
		Relative to BW (%)	0.012 ± 0.004	0.013 ± 0.006	0.012 ± 0.001	0.013 ± 0.003	0.014 ± 0.002	0.014 ± 0.001
	Uterus w/ cervix	Absolute (gram)	5.334 ± 0.529	4.757 ± 0.678	3.514 ± 1.266	461 ± 1.400	6.295 ± 1.910	4.341 ± 3.395
		Relative to BW (%)	0.196 ± 0.024	0.167 ± 0.045	0.149 ± 0.051	0.170 ± 0.065	0.217 ± 0.068	0.168 ± 0.128

Data are presented as mean ± SD.
Abbreviations: BW (bodyweight); n (number of animals).

Table S22A. Histopathology in male cynomolgus monkeys

Histopathological findings		14 day follow-up (n=3 per group)				Recovery animals (n=2 per group)	
		Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day	Vehicle	400 mg/kg/day
Adrenal glands	No visible lesions	n=2	n=3	n=2	n=3	n=2	n=1
	Eosinophelia with decreased vacuolation; diffuse	n=0	n=0	n=0	n=0	n=0	n=1
	... moderate	n=0	n=0	n=0	n=0	n=0	n=1
	Eosinophilia with decreased vacuolation; focal	n=0	n=0	n=1	n=0	n=0	n=0
	... minimal	n=0	n=0	n=1	n=0	n=0	n=0
	Mineralization	n=1	n=0	n=0	n=0	n=0	n=0
	... minimal	n=1	n=0	n=0	n=0	n=0	n=0
Aorta	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Bone marrow, sternum	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Bone, femur	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Bone, sternum	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Brain	No visible lesions	n=3	n=2	n=3	n=2	n=2	n=2
	Infiltration, mononuclear cells	n=0	n=1	n=0	n=1	n=0	n=0
	... minimal	n=0	n=1	n=0	n=1	n=0	n=0
Epididymides	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Esophagus	No visible lesions	n=3	n=3	n=1	n=1	n=2	n=2
	Infiltration, mixed inflammatory cells	n=0	n=0	n=0	n=1	n=0	n=0
	... minimal	n=0	n=0	n=0	n=1	n=0	n=0
	Infiltration, mononuclear cells	n=0	n=0	n=2	n=1	n=0	n=0
	... minimal	n=0	n=0	n=2	n=1	n=0	n=0
Eyes	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Gallbladder	No visible lesions	n=1	n=1	n=3	n=2	n=1	n=1
	Infiltration, mixed inflammatory cells	n=2	n=1	n=0	n=0	n=0	n=0
	... minimal	n=2	n=1	n=0	n=0	n=0	n=0
	Infiltration, mononuclear cells	n=0	n=1	n=0	n=1	n=1	n=1
	... minimal	n=0	n=1	n=0	n=1	n=1	n=1
Heart	No visible lesions	n=1	n=2	n=2	n=2	n=1	n=1
	Infiltration, mixed inflammatory cells	n=2	n=1	n=0	n=0	n=0	n=0
	... minimal	n=2	n=1	n=0	n=0	n=0	n=0
	Infiltration, mononuclear cells	n=0	n=0	n=1	n=1	n=1	n=1
	... minimal	n=0	n=0	n=1	n=1	n=1	n=1
Injection sites	No visible lesions	n=0	n=0	n=1	n=1	n=2	n=0
	Hemorrhage	n=2	n=3	n=2	n=2	n=0	n=0
	... mild	n=0	n=1	n=0	n=0	n=0	n=0
	... moderate	n=2	n=2	n=2	n=1	n=0	n=0
	... marked	n=0	n=0	n=0	n=1	n=0	n=0
	Infiltration, mixed inflammatory cells	n=1	n=3	n=1	n=1	n=0	n=0
	... mild	n=1	n=3	n=1	n=1	n=0	n=0
	Infiltration, mononuclear cells	n=2	n=1	n=0	n=0	n=0	n=2
	... minimal	n=2	n=1	n=0	n=0	n=0	n=2
Intestine, cecum	No visible lesions	n=3	n=2	n=3	n=3	n=1	n=0
	Parasite; protozoa	n=0	n=1	n=0	n=0	n=0	n=2
	Hyperplasia, germinal centers	n=0	n=0	n=0	n=0	n=1	n=0
	... mild	n=0	n=0	n=0	n=0	n=1	n=0
Intestine, colon	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=1
	Parasite; protozoa	n=0	n=0	n=0	n=0	n=0	n=1
Intestine, duodenum	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2

Table S22A. continued.

Histopathological findings		14 day follow-up (n=3 per group)				Recovery animals (n=2 per group)	
		Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day	Vehicle	400 mg/kg/day
Intestine, ileum	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Intestine, jejunum	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Intestine, rectum	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Kidneys	No visible lesions	n=1	n=1	n=0	n=1	n=1	n=0
	Infiltration, mononuclear cells	n=2	n=2	n=3	n=2	n=1	n=2
	... minimal	n=2	n=2	n=3	n=2	n=1	n=2
	Mineralization	n=0	n=0	n=1	n=0	n=1	n=0
	... minimal	n=0	n=0	n=1	n=0	n=1	n=0
	Degeneration/regeneration	n=1	n=0	n=0	n=0	n=0	n=0
	... minimal	n=1	n=0	n=0	n=0	n=0	n=0
Liver	No visible lesions	n=2	n=3	n=1	n=1	n=0	n=1
	Infiltration, mononuclear cells	n=1	n=0	n=2	n=2	n=2	n=1
	... minimal	n=1	n=0	n=2	n=2	n=2	n=1
Lungs	No visible lesions	n=3	n=3	n=2	n=2	n=1	n=2
	Infiltration, mononuclear cells	n=0	n=0	n=1	n=0	n=1	n=0
	... minimal	n=0	n=0	n=1	n=0	n=1	n=0
	Foreign material	n=0	n=0	n=0	n=1	n=0	n=0
Lymph node, mesenteric	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Lymph node, mandibular	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Nerves, optic	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Nerve, sciatic	No visible lesions	n=3	n=2	n=3	n=3	n=2	n=2
	Perivascular; infiltration, mononuclear cells	n=0	n=1	n=0	n=0	n=0	n=0
	... minimal	n=0	n=1	n=0	n=0	n=0	n=0
Pancreas	No visible lesions	n=2	n=3	n=3	n=2	n=1	n=2
	Infiltration, mononuclear cells	n=1	n=0	n=0	n=1	n=1	n=0
	... minimal	n=1	n=0	n=0	n=1	n=1	n=0
Parathyroid gland	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=1
	Ectopic thymus	n=1	n=0	n=0	n=0	n=0	n=0
	Infiltration, mononuclear cells	n=0	n=0	n=0	n=0	n=0	n=1
	... minimal	n=0	n=0	n=0	n=0	n=0	n=1
Pituitary gland	No visible lesions	n=3	n=2	n=3	n=3	n=2	n=2
	Cyst	n=0	n=1	n=0	n=0	n=0	n=0
	... mild	n=0	n=1	n=0	n=0	n=0	n=0
Prostate gland	No visible lesions	n=2	n=2	n=1	n=2	n=0	n=1
	Infiltration, mononuclear cells	n=1	n=1	n=2	n=1	n=2	n=1
	... minimal	n=1	n=1	n=2	n=1	n=2	n=1
Salivary gland, submandibular	No visible lesions	n=1	n=0	n=1	n=2	n=0	n=1
	Infiltration, mononuclear cells	n=2	n=3	n=2	n=1	n=2	n=1
	... minimal	n=2	n=3	n=2	n=1	n=2	n=1
Seminal vesicles	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=1
	Infiltration, mononuclear cells	n=0	n=0	n=0	n=0	n=0	n=1
	... minimal	n=0	n=0	n=0	n=0	n=0	n=1
Skeletal muscle	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Skin	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2

Table S22A. continued.

Histopathological findings		14 day follow-up (n=3 per group)				Recovery animals (n=2 per group)	
		Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day	Vehicle	400 mg/kg/day
Spinal cord, thoracic	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Spleen	No visible lesions	n=2	n=3	n=3	n=3	n=2	n=2
	Hyperplasia, germinal centers	n=1	n=0	n=0	n=0	n=0	n=0
	... mild	n=1	n=0	n=0	n=0	n=0	n=0
Stomach	No visible lesions	n=0	n=1	n=0	n=0	n=1	n=0
	Infiltration, mixed inflammatory cells	n=3	n=0	n=0	n=3	n=0	n=0
	... minimal	n=0	n=0	n=0	n=1	n=0	n=0
	... mild	n=3	n=0	n=0	n=2	n=0	n=0
	Hyperplasia, germinal centers	n=0	n=2	n=3	n=0	n=1	n=2
	... minimal	n=0	n=1	n=2	n=0	n=1	n=0
	... mild	n=1	n=1	n=1	n=0	n=0	n=2
Testes	No visible lesions	n=0	n=1	n=0	n=3	n=0	n=0
	Fibroplasia	n=1	n=1	n=1	n=0	n=0	n=0
	... mild	n=1	n=1	n=1	n=0	n=0	n=0
	Immature but normal	n=3	n=2	n=3	n=0	n=2	n=2
Thymus	No visible lesions	n=3	n=3	n=3	n=2	n=2	n=2
	Involution	n=0	n=0	n=0	n=1	n=0	n=0
	... mild	n=0	n=0	n=0	n=1	n=0	n=0
Thyroid gland	No visible lesions	n=3	n=0	n=1	n=2	n=1	n=2
	Ectopic thymus	n=0	n=1	n=2	n=0	n=1	n=0
	Infiltration, macrophages	n=0	n=1	n=0	n=0	n=1	n=0
	... minimal	n=0	n=1	n=0	n=0	n=1	n=0
	Infiltration, mononuclear cells	n=0	n=2	n=1	n=1	n=1	n=0
	... minimal	n=0	n=2	n=1	n=1	n=1	n=0
Tongue	No visible lesions	n=3	n=1	n=2	n=3	n=1	n=1
	Not examined, no section	n=0	n=0	n=0	n=0	n=0	n=1
	Infiltration, mixed inflammatory cells	n=0	n=0	n=1	n=0	n=0	n=0
	... mild	n=0	n=0	n=1	n=0	n=0	n=0
	Infiltration, mononuclear cells	n=0	n=2	n=0	n=0	n=1	n=0
	... minimal	n=0	n=2	n=0	n=0	n=1	n=0
Trachea	No visible lesions	n=2	n=2	n=1	n=1	n=1	n=1
	Infiltration, mononuclear cells	n=1	n=1	n=2	n=2	n=1	n=1
	... minimal	n=1	n=1	n=2	n=2	n=0	n=1
	... mild	n=0	n=0	n=0	n=0	n=1	n=0
Urinary bladder	No visible lesions	n=2	n=2	n=3	n=1	n=1	n=0
	Infiltration, mononuclear cells	n=2	n=2	n=3	n=1	n=1	n=2
	... minimal	n=2	n=2	n=3	n=1	n=1	n=2

Abbreviations: n (number of animals).

Table S22B. Histopathology in female cynomolgus monkeys.

Histopathological findings		14 day follow-up (n=3 per group)				Recovery animals (n=2 per group)	
		Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day	Vehicle	400 mg/kg/day
Adrenal glands	No visible lesions	n=3	n=3	n=3	n=2	n=1	n=2
	Mineralization	n=0	n=0	n=0	n=1	n=1	n=0
	... minimal	n=0	n=0	n=0	n=1	n=1	n=0
Aorta	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Bone marrow, sternum	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Bone, femur	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Bone, sternum	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Brain	No visible lesions	n=2	n=2	n=2	n=3	n=2	n=2
	Infiltration, mononuclear cells	n=1	n=1	n=1	n=0	n=0	n=0
	... minimal	n=1	n=1	n=1	n=0	n=0	n=0
Cervix	No visible lesions	n=3	n=3	n=2	n=33	n=2	n=2
	Infiltration, mononuclear cells	n=0	n=0	n=1	n=0	n=0	n=0
	... minimal	n=0	n=0	n=1	n=0	n=0	n=0
Esophagus	No visible lesions	n=2	n=2	n=2	n=3	n=1	n=2
	Infiltration, mononuclear cells	n=1	n=1	n=1	n=0	n=1	n=0
	... minimal	n=0	n=1	n=1	n=0	n=1	n=0
	... mild	n=1	n=0	n=0	n=0	n=0	n=0
Eyes	No visible lesions	n=3	n=3	n=3	n=3	n=1	n=2
	Iris; infiltration, mononuclear cells	n=0	n=0	n=0	n=0	n=1	n=0
	... minimal	n=0	n=0	n=0	n=0	n=1	n=0
Gallbladder	No visible lesions	n=1	n=2	n=1	n=2	n=2	n=1
	Infiltration, mixed inflammatory cells	n=0	n=1	n=0	n=1	n=0	n=0
	... minimal	n=0	n=1	n=0	n=1	n=0	n=0
	Infiltration, mononuclear cells	n=2	n=0	n=2	n=0	n=0	n=1
	... minimal	n=2	n=0	n=2	n=0	n=0	n=1
Heart	No visible lesions	n=2	n=2	n=1	n=1	n=1	n=1
	Infiltration, mixed inflammatory cells	n=1	n=0	n=0	n=2	n=0	n=0
	... minimal	n=1	n=0	n=0	n=2	n=0	n=0
	Infiltration, mononuclear cells	n=0	n=1	n=2	n=0	n=1	n=1
	... minimal	n=0	n=1	n=2	n=0	n=1	n=1
Injection sites	No visible lesions	n=1	n=1	n=0	n=1	n=1	n=1
	Hemorrhage	n=1	n=2	n=2	n=1	n=0	n=0
	... mild	n=0	n=1	n=1	n=0	n=0	n=0
	... moderate	n=1	n=1	n=1	n=1	n=0	n=0
	Infiltration, mixed inflammatory cells	n=0	n=2	n=2	n=1	n=0	n=0
	... mild	n=0	n=0	n=1	n=0	n=0	n=0
	... mild	n=0	n=2	n=1	n=1	n=0	n=0
	Infiltration, mononuclear cells	n=1	n=0	n=0	n=1	n=1	n=1
	... minimal	n=1	n=0	n=0	n=1	n=1	n=1
Intestine, cecum	No visible lesions	n=3	n=2	n=3	n=3	n=1	n=2
	Parasite; protozoa	n=0	n=1	n=0	n=0	n=1	n=0
	Glands; infiltration, mixed inflammatory cells	n=0	n=1	n=0	n=0	n=0	n=0
	... minimal	n=0	n=1	n=0	n=0	n=0	n=0
Intestine, colon	No visible lesions	n=3	n=2	n=3	n=3	n=2	n=2
	Glands; infiltration, mixed inflammatory cells	n=0	n=1	n=0	n=0	n=0	n=0
	... minimal	n=0	n=1	n=0	n=0	n=0	n=0
Intestine, duodenum	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2

Table S22B. continued.

Histopathological findings		14 day follow-up (n=3 per group)				Recovery animals (n=2 per group)	
		Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day	Vehicle	400 mg/kg/day
Intestine, ileum	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Intestine, jejunum	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Intestine, rectum	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Kidneys	No visible lesions	n=1	n=0	n=0	n=0	n=0	n=0
	Pelvis; infiltration, mixed inflammatory cells	n=0	n=0	n=0	n=1	n=0	n=0
	... mild	n=0	n=0	n=0	n=1	n=0	n=0
	Infiltration, mononuclear cells	n=2	n=3	n=3	n=2	n=2	n=2
	... minimal	n=2	n=3	n=3	n=2	n=2	n=2
Liver	No visible lesions	n=1	n=0	n=0	n=2	n=1	n=0
	Infiltration, mononuclear cells	n=2	n=3	n=3	n=1	n=1	n=2
	... minimal	n=2	n=3	n=3	n=1	n=1	n=2
Lungs	No visible lesions	n=2	n=3	n=2	n=3	n=1	n=2
	Infiltration, macrophages	n=1	n=0	n=0	n=0	n=0	n=0
	... minimal	n=1	n=0	n=0	n=0	n=0	n=0
	Infiltration, mononuclear cells	n=0	n=0	n=1	n=0	n=1	n=0
	... minimal	n=0	n=0	n=1	n=0	n=1	n=0
Lymph node, mesenteric	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Lymph node, mandibular	No visible lesions	n=2	n=3	n=2	n=3	n=2	n=2
	Hematopoiesis; increased	n=1	n=0	n=0	n=0	n=0	n=0
	... mild	n=1	n=0	n=0	n=0	n=0	n=0
	Sinus plasmacytosis	n=0	n=0	n=1	n=0	n=0	n=0
	... mild	n=0	n=0	n=1	n=0	n=0	n=0
Mammary gland	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Nerves, optic	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Nerve, sciatic	No visible lesions	n=3	n=3	n=3	n=3	n=1	n=2
	Perivascular; infiltration, mononuclear cells	n=0	n=0	n=0	n=0	n=1	n=0
	... minimal	n=0	n=0	n=0	n=0	n=1	n=0
Ovaries	No visible lesions	n=1	n=2	n=2	n=1	n=1	n=2
	Mineralization	n=2	n=1	n=1	n=2	n=1	n=0
	... minimal	n=2	n=1	n=1	n=2	n=1	n=0
Pancreas	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Parathyroid gland	No visible lesions	n=3	n=3	n=2	n=3	n=1	n=1
	Ectopic thymus	n=0	n=0	n=1	n=0	n=1	n=0
	Cyst	n=0	n=0	n=0	n=0	n=0	n=1
	... minimal	n=0	n=0	n=0	n=0	n=0	n=1
Pituitary gland	No visible lesions	n=2	n=3	n=3	n=3	n=2	n=2
	Cyst	n=1	n=0	n=0	n=0	n=0	n=0
	... minimal	n=1	n=0	n=0	n=0	n=0	n=0
	Infiltration, mononuclear cells	n=1	n=0	n=0	n=0	n=0	n=0
	... minimal	n=1	n=0	n=0	n=0	n=0	n=0
Salivary gland, submandibular	No visible lesions	n=2	n=1	n=1	n=1	n=1	n=0
	Infiltration, mononuclear cells	n=1	n=2	n=2	n=2	n=1	n=2
	... minimal	n=1	n=2	n=2	n=2	n=1	n=2
Skeletal muscle	No visible lesions	n=3	n=2	n=2	n=3	n=1	n=1
	Infiltration, mononuclear cells	n=0	n=1	n=1	n=0	n=1	n=1
	... minimal	n=0	n=1	n=1	n=0	n=1	n=1

Table S22B. continued

Histopathological findings		14 day follow-up (n=3 per group)				Recovery animals (n=2 per group)	
		Vehicle	25 mg/kg/day	50 mg/kg/day	100 mg/kg/day	Vehicle	400 mg/kg/day
Skin	No visible lesions	n=3	n=2	n=2	n=3	n=2	n=2
	Infiltration, mixed inflammatory cells	n=0	n=1	n=0	n=0	n=0	n=0
	... minimal	n=0	n=1	n=0	n=0	n=0	n=0
	Infiltration, mononuclear cells	n=0	n=0	n=1	n=0	n=0	n=0
	... minimal	n=0	n=0	n=1	n=0	n=0	n=0
Spinal cord, thoracic	No visible lesions	n=3	n=3	n=3	n=3	n=2	n=2
Spleen	No visible lesions	n=3	n=3	n=3	n=3	n=1	n=2
	Hyperplasia, germinal centers	n=0	n=0	n=0	n=0	n=1	n=0
	... mild	n=0	n=0	n=0	n=0	n=1	n=0
Stomach	No visible lesions	n=0	n=2	n=1	n=0	n=0	n=0
	Infiltration, mixed inflammatory cells	n=2	n=0	n=0	n=3	n=0	n=0
	... minimal	n=0	n=0	n=0	n=1	n=0	n=0
	... mild	n=2	n=0	n=0	n=2	n=0	n=0
	Infiltration, mononuclear cells	n=1	n=0	n=0	n=0	n=0	n=0
	... mild	n=1	n=0	n=0	n=0	n=0	n=0
	Hyperplasia, germinal centers	n=0	n=1	n=2	n=0	n=2	n=2
	... minimal	n=0	n=1	n=0	n=0	n=1	n=1
	... mild	n=0	n=0	n=2	n=0	n=1	n=1
Thymus	No visible lesions	n=2	n=3	n=2	n=3	n=2	n=2
	Cyst	n=1	n=0	n=0	n=0	n=0	n=0
	... minimal	n=1	n=0	n=0	n=0	n=0	n=0
	Involution	n=1	n=0	n=1	n=0	n=0	n=0
	... mild	n=1	n=0	n=1	n=0	n=0	n=0
Thyroid gland	No visible lesions	n=3	n=2	n=1	n=1	n=1	n=0
	Cyst	n=0	n=0	n=0	n=1	n=0	n=1
	... mild	n=0	n=0	n=0	n=1	n=0	n=1
	Infiltration, macrophages	n=0	n=1	n=1	n=0	n=0	n=1
	... minimal	n=0	n=1	n=1	n=0	n=0	n=1
	Infiltration, mononuclear cells	n=0	n=0	n=1	n=2	n=1	n=1
	... minimal	n=0	n=0	n=1	n=2	n=1	n=1
Tongue	No visible lesions	n=2	n=2	n=3	n=2	n=0	n=2
	Infiltration, mononuclear cells	n=1	n=1	n=0	n=1	n=2	n=0
	... minimal	n=1	n=1	n=0	n=1	n=1	n=0
Trachea	No visible lesions	n=1	n=0	n=3	n=2	n=1	n=1
	Infiltration, mononuclear cells	n=2	n=3	n=0	n=1	n=1	n=1
	... minimal	n=2	n=3	n=0	n=1	n=0	n=1
	... mild	n=0	n=0	n=0	n=0	n=1	n=0
Urinary bladder	No visible lesions	n=1	n=0	n=3	n=2	n=2	n=2
	Infiltration, mononuclear cells	n=2	n=3	n=0	n=1	n=0	n=0
	... minimal	n=2	n=3	n=0	n=1	n=0	n=0
Uterus	No visible lesions	n=3	n=3	n=2	n=3	n=2	n=2
	Infiltration, mononuclear cells	n=0	n=0	n=1	n=0	n=0	n=0
	... minimal	n=0	n=0	n=1	n=0	n=0	n=0
Vagina	No visible lesions	n=0	n=0	n=0	n=0	n=0	n=0
	Infiltration, mononuclear cells	n=3	n=3	n=3	n=3	n=2	n=2
	... minimal	n=1	n=1	n=3	n=2	n=0	n=1
	... mild	n=2	n=2	n=0	n=1	n=2	n=1

Abbreviations: n (number of animals).

Table S23. Toxicokinetics of Adrecizumab in cynomolgus monkeys

Gender	Parameter	25 mg/kg/day		50 mg/kg/day		100 mg/kg/day	
		Day 1[A]	Day 14	Day 1	Day 14[B]	Day 1[C]	Day 14[D]
Male	No. of animals	n=3	n=3	n=3	n=3	n=5	n=5
	T_{max} (h)	0.517 ± 0.00	0.517 ± 0.00	0.517 ± 0.00	0.517 ± 0.00	0.517 ± 0.00	0.517 ± 0.00
	C_{max} (µg/mL)	478 ± 19.8	732 ± 61.8	948 ± 161	1560 ± 398	2150 ± 440	3620 ± 635
	AUC_{0-24h} (h* µg/mL)	6110 ± 88.9	11270 ± 493	11700 ± 702	23200 ± 3390	28800 ± 3410	52300 ± 5850
	AUC_{0-inf} (h* µg/mL)[E]	23700 ± 1400	28700 ± 500	42200 ± 3500	68900 ± 14400	103200 ± 18500	184000 ± 67600
	$T_{n=1}$ (h)	66.8 ± 0.862	41.2 ± 4.31	66.8 ± 0.862	41.2 ± 4.31	49.5 ± 9.12	-
Female	No. of animals	n=3	n=3	n=3	n=3	n=5	n=5
	T_{max} (h)	0.517 ± 0.00	0.517 ± 0.00	0.517 ± 0.00	0.517 ± 0.00	0.517 ± 0.00	0.517 ± 0.00
	C_{max} (µg/mL)	493 ± 63.3	797 ± 177	944 ± 90.9	1650 ± 488	2120 ± 647	3530 ± 183
	AUC_{0-24h} (h* µg/mL)	6880 ± 1770	12610 ± 3500	12470 ± 153	26400 ± 4530	23900 ± 7630	48000 ± 7470
	AUC_{0-inf} (h*µg/mL)	28800 ± 8590	40300 ± 27200	50400 ± 2890	78000 ± 33900	84200 ± 22600	185400 ± 103500
	$T_{n=1}$ (h)	68.9 ± 12.0	437 ± 31.5	68.9 ± 12.0	42.2 ± 16.12	51.3 ± 10.4	-
Male & Female	No. of animals	n=6	n=6	n=6	n=6	n=10	n=10
	T_{max} (h)	0.517 ± 0.00	0.517 ± 0.00	0.517 ± 0.00	0.571 ± 0.00	0.517 ± 0.00	0.517 ± 0.00
	C_{max} (µg/mL)	487 ± 46.5	764 ± 124	946 ± 117	1590 ± 376	2140 ± 504	3570 ± 413
	AUC_{0-24h} (h* µg/mL)	6500 ± 1200	11940 ± 2360	12100 ± 631	24480 ± 3730	26400 ± 6150	50100 ± 6720
	AUC_{0-inf} (h* µg/mL)	26300 ± 6160	34500 ± 18340	46300 ± 5350	72500 ± 20420	93700 ± 21900	184700 ± 82400
	$T_{n=1}$ (h)	78.2 ± 6.28	38.2 ± 14.1	67.8 ± 7.68	42.2 ± 16.12	51.3 ± 10.4	-
	$AUC_{0-24h/dose}$ (h*kg* µg/mL/mg)	260 ± 48.0	478 ± 94.3	241 ± 12.6	490 ± 74.6	264 ± 5.04	501 ± 67.2
	$C_{max/dose}$ (kg* µg/mL/mg)	19.5 ± 1.88	30.6 ± 4.95	18.9 ± 2.33	31.9 ± 7.51	21.4 ± 5.04	35.7 ± 4.13
	R_{acc}	-	1.84 ± 0.101	-	2.04 ± 0.227	-	2.00 ± 2.04

Data are presented as mean ± SD

[A] 1 male animal was excluded as an outlier for T_{max} and C_{max}

[B] 1 female animal was excluded from the evaluation (dosing problem)

[C] 1 female animal was excluded as an outlier for T_{max} and C_{max}

[D] 1 male animal was excluded as an outlier for T_{max} and C_{max}

[E] AUC0-inf, high uncertainty because extrapolation to infinity > 20% or weak regression

- not calculated

Racc(0-24h) = AUC(0-24h) on Day 14 / AUC(0-24h) on Day 1

Part IV

Clinical evaluation of
the adrenomedullin-binding antibody
Adrecizumab

Chapter 12

Safety, tolerability and pharmacokinetics/dynamics of the adrenomedullin antibody Adrecizumab in a first-in-human study and during experimental human endotoxemia in healthy subjects

Christopher Geven, Dirk van Lier, Alice Blet, Roel Peelen, Bas ten Elzen, Alexandre Mebazaa, Matthijs Kox and Peter Pickkers

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Abstract

Aims

Adrenomedullin (ADM) is an important regulator of endothelial barrier function and vascular tone, and may represent a novel treatment target in sepsis. The non-neutralizing ADM antibody Adrecizumab has shown promising results in preclinical sepsis models. In the present study, we investigated the safety, tolerability and pharmacokinetics (PK)/pharmacodynamics of Adrecizumab in a first-in-man study and in a second study during experimental human endotoxemia.

Methods

Forty-eight healthy male volunteers were enrolled in two randomized, double-blind, placebo-controlled phase I studies. In both studies, subjects received placebo or one of three doses of Adrecizumab (n = 6 per group). In the second study, a bolus of 1 ng/kg endotoxin was followed by infusion of 1 ng/kg/h endotoxin for 3 h to induce systemic inflammation, and the study medication infusion started 1 h after endotoxin bolus administration.

Results

Adrecizumab showed an excellent safety profile in both studies. PK analyses showed proportional increases in the maximum plasma concentration of Adrecizumab with increasing doses, a small volume of distribution, a low clearance rate and a terminal half-life of ~14 days. Adrecizumab elicited a pronounced increase in plasma ADM levels, whereas levels of mid-regional proadrenomedullin remained unchanged, indicating that de novo synthesis of ADM was not influenced. In the second study, no effects of Adrecizumab on cytokine clearance were observed, whereas endotoxin-induced flu-like symptoms resolved more rapidly.

Conclusions

Administration of adrecizumab is safe and well tolerated in humans, both in the absence and presence of systemic inflammation. These findings pave the way for further investigation of Adrecizumab in sepsis patients.

Introduction

Sepsis is a major health problem for patients with infectious diseases worldwide, with increasing incidence and a high mortality rate[1-3]. It is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection[4]. Sepsis-induced vascular effects include vasodilation and loss of vascular barrier function[5]. This results in hypotension, tissue oedema and ultimately, lethal organ dysfunction. Besides (supportive) therapies such as antibiotics, mechanical ventilation and vasopressors, there are currently no adjuvant therapies available.

Adrenomedullin (ADM) is a free circulating peptide hormone, which is involved in the regulation of vascular tone and stabilization of the endothelial barrier[6-8]. During sepsis and most pronounced during septic shock, elevated concentrations of circulating ADM are observed, which correlate with disease severity and mortality[9,10]. However, correlation does not imply causation, and increased levels of ADM could also represent a (failing) compensatory response. Mechanistic studies actually indicate that ADM can exert both beneficial and detrimental effects in sepsis. Therefore, ADM is referred to as a 'double-edged' sword in sepsis. On one hand, preclinical studies in animal models of systemic inflammation and sepsis have shown that ADM administration restores vascular barrier function through effects on endothelial cells, thereby reducing detrimental tissue oedema[11-14]. On the other hand, ADM has also been reported to induce vasodilation and hypotension[15-17], which could in theory further aggravate hypotension in patients with septic shock. It was thus hypothesized that modulation of ADM with antibodies could be beneficial, if it would retain or even potentiate the beneficial effects of ADM while negating its potentially detrimental vasodilatory effects. Interestingly, a highly specific non-neutralizing mouse monoclonal antibody (HAM1101) was previously shown to improve survival in cecal ligation and puncture (CLP)-induced sepsis in mice[18]. In addition, in a fully resuscitated murine CLP-induced septic shock model, treatment with this antibody resulted in reduced vasopressor demand and improved organ function[19]. These promising results led to the development of a humanized antibody for further clinical investigation (HAM8101, later named Adrecizumab). In lipopolysaccharide (LPS)-induced systemic inflammation in rats and CLP-induced sepsis in mice, Adrecizumab attenuated vascular leakage and vascular dysfunction, as well as improved survival[20]. Extensive preclinical safety and toxicological studies did not reveal any safety concerns (unpublished data).

The present work describes two phase I studies in which the first-in-human

safety, tolerability and pharmacokinetics (PK) and pharmacodynamics (PD) of single, escalating intravenous doses of Adrecizumab were investigated. The first study was conducted in healthy male volunteers during normal non-inflammatory conditions. The second study was conducted during systemic inflammation evoked by experimental human endotoxemia. The experimental endotoxemia model is a safe and reproducible method for inducing a controlled transient systemic inflammatory response in humans by intravenous administration of E. coli endotoxin (LPS)[21].

Methods

General

Firstly, a first-in-human phase I, randomized, double-blind, placebo-controlled study was conducted to evaluate single escalating intravenous (i.v.) doses of Adrecizumab in healthy male subjects. Next, a second phase I, randomized, double-blind, placebo-controlled study was conducted to evaluate single escalating i.v. doses of Adrecizumab in healthy male subjects during experimental human endotoxemia (details provided below). Both studies were conducted at a single site (the Department of Intensive Care Medicine at the Radboud University Medical Center in Nijmegen, the Netherlands), and were carried out in accordance with the Declaration of Helsinki and Good Clinical Practice standards. The study protocols were approved by the local ethics committee of the Radboud University Medical Center (approval numbers 2016–2283 and 2016–2740) prior to recruitment and inclusion of subjects, and registered at clinicaltrials.gov (NTC02991508 and NTC03083171).

Study medication

Adrecizumab is a non-neutralizing humanized high-affinity immunoglobulin (Ig) G1κ full-length antibody directed against the N-terminus of ADM. Adrecizumab was produced using Chinese hamster ovary cells under good manufacturing practice conditions. Adrecizumab and placebo were supplied by the study sponsor (Adrenomed AG, Hennigsdorf, Germany) as a solution for injection in identical sterile single-use vials, containing 10.4 ml solution, allowing for an extractable volume of 10 ml. Adrecizumab (20 mg/ml) was dissolved in a vehicle consisting of histidine-hydrochloride monochloride, glycine and water. The placebo solution consisted of the identical vehicle. Vials were manufactured by Glycotope Biotechnology GmbH (Heidelberg,

Germany). Manufacturing, packaging, quality control and preparation were described in an Investigational Medicinal Product Dossier. Randomization of subjects (using a predetermined randomization list) and preparation of study medication was performed by an independent and unblinded research team, who were not involved in any other aspect of the studies. Dose selection for the present human studies (0.5, 2.0 and 8.0 mg/kg Adrecizumab) was based on preclinical data, showing a no adverse events level (NOAEL) of 400 mg/kg and 100 mg/kg for rats and cynomolgus monkeys, respectively. Using a conversion factor of 6.2 for the rat and 3.1 for the monkey, the human equivalent doses are 65 mg/kg and 32 mg/kg, providing a safety margin factor of 130 or 64 to the proposed starting dose of 0.5 mg/kg. In addition, a therapeutic dose of 2.0 mg/kg was regarded as sufficient, based on various preclinical efficacy studies[18-20] (and other, as yet unpublished, data). Including an additional higher dose of 8.0 mg/kg in humans provides the opportunity to demonstrate potential dose dependency of this higher dosage.

Subjects

After giving written consent, male subjects, aged 18–35 years, with a body mass index (BMI) between 18 kg/m^2 and 30 kg/m^2 were included. Before participation, health status was determined by past medical history, physical examination, 12-lead electrocardiography (ECG) and safety laboratory tests at a screening visit. Exclusion criteria included atopic constitution, use of any medication, flu-like symptoms 14 days prior to the studies, significant blood loss and/or participation in any other clinical trial within 90 days prior to the studies. For the experimental endotoxemia study, previous participation in endotoxemia trials was an additional exclusion criterion. The use of recreational drugs was prohibited 7 days prior to and during the study and in the subsequent 90-day follow-up period. Alcohol and tobacco use was prohibited 24 h before and after the experimental day.

Study procedures

The general study procedures are depicted in **Figure 1** and were virtually identical for both studies, except for the administration of LPS in the endotoxemia study. Fasted subjects were admitted to the research unit in the morning (07:30 h). An i.v. cannula was placed for infusion of fluids, as well as administration of endotoxin and study drug. An intra-arterial cannula was placed for continuous blood pressure monitoring and frequent blood withdrawal. Vital signs were continuously monitored until discharge. In each

study, 24 eligible subjects were assigned to one of three dose groups: 0.5, 2 and 8 mg/kg body weight. Each dose group consisted of eight subjects randomly assigned to receive either Adrecizumab or placebo (n = 6 active study drug, n = 2 placebo). Frequent blood samples were collected during the first 8 h after study drug administration, for analyses of safety laboratory parameters as well as PK and PD parameters. Subjects were discharged 8 h after study drug administration, after assessment and confirmation of their fitness by the investigator. Owing to the expected long half-life ($T_{½}$) of Adrecizumab, subjects returned for further follow-up visits after 1, 7, 14, 28, 60 and 90 days.

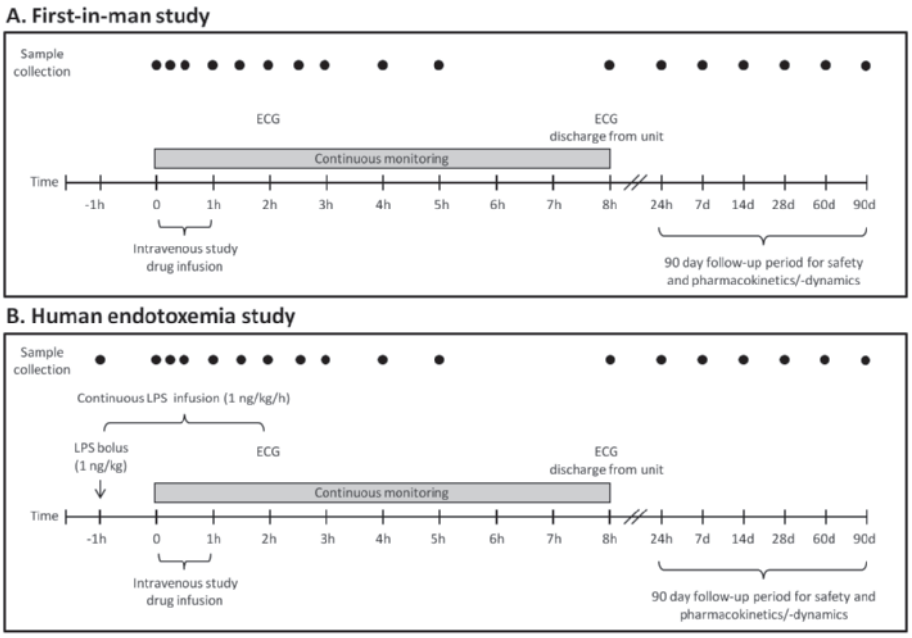

Figure 1. Schematic overview of study procedures for the first-in-human study (**A**) and the human endotoxemia study (**B**). *Abbreviations: ECG, electrocardiography; LPS, lipopolysaccharide.*

Induction of systemic inflammation in the human endotoxemia model

In the present study, an endotoxemia model, applying continuous infusion of LPS for 3 h, was used because this is thought to be a better representation of the inflammatory response as observed in patients with sepsis than a bolus administration of LPS[22]. Systemic inflammation was induced by a bolus administration of 1 ng/kg Escherichia coli type O113 LPS (List Biological Laboratories Inc., Campbell, CA, USA) 1 h prior to study drug administration. This bolus was followed by a continuous intravenous infusion of LPS 1 ng/kg/h for 3 h to induce systemic inflammation. To prevent vasovagal responses, subjects received prehydration with 1.5 L glucose 2.5%/NaCl 0.45% over the course of 45 min[23].

Safety parameters

Safety and tolerability were the primary endpoints of both studies. Frequent safety and tolerability assessments were performed on the study drug administration day for both studies until discharge and during the 90-day follow-up period. Safety parameters included blood pressure, heart rate and peripheral oxygen saturation (recorded from a Philips MP50 patient monitor [Philips, Eindhoven, the Netherlands]; on the study drug administration day, data were sampled every 30 s by a custom inhouse-developed data recording system), temperature (FirstTemp Genius 2; Sherwood Medical, St Louis, MO, USA), 12-lead ECG (Philips PageWriter Trim III, Philips, Amsterdam, the Netherlands) and routine haematology and biochemistry laboratory tests. An adverse event (AE) was defined as any untoward medical occurrence in a clinical trial subject administered a study product, which did not necessarily have a causal relationship with this treatment. AEs were recorded throughout the study and follow-up period. All AEs were judged by the investigator with regard to severity (mild, moderate or severe) and their relation to the study drug (unrelated, possible, probably or definite). In the human endotoxemia study, common symptoms of endotoxemia (headache, abdominal pain, back pain, fever and muscle aches) were not regarded as AEs unless they were of abnormal severity or duration. In order to minimize the risks for subjects in both studies, dosage groups were tested sequentially if the previous dose was tolerated without relevant side effects. In each dose group, the first four subjects were tested consecutively, with 48 h between experimental days. In both studies, an independent data safety monitoring board reviewed safety data, including vital signs, laboratory parameters and AEs, and approved to continue the study with the next dose groups.

Sample collection

Blood was drawn in ethylenediaminetetraacetic acid anticoagulated vacutainers and centrifuged (10 min, 2000 g, 4°C), after which plasma was stored at -80°C until analysis.

Pharmacokinetic analysis

For both studies, PK analysis was performed on samples collected at the following time points relative to study drug administration: immediately prior to administration and then 15, 30, 60 and 90 min; 2, 3, 4 and 8 h; and 1, 7, 14, 28, 60 and 90 days after administration. PK analysis was performed by Aurigon Toxicological Research Center (Dunakeszi, Hungary) under good laboratory practice conditions. Free (unbound) Adrecizumab was quantified in the human plasma samples using a validated luminescence immunoassay, with a quantification limit of 0.85 μg/ml. The highest observed plasma concentration was defined as C_{max}. The area under the plasma concentration–time curve from t0 to the last measurement (AUC_{0-t}) was calculated using the linear trapezoidal rule. The elimination rate constant (λ_z) was calculated by log linear regression of concentrations observed during the terminal phase of elimination. The AUC from t0 to infinity ($AUC_{0-\infty}$) was calculated as the sum of AUC_{0-t} and the extrapolated area using the last measured concentration ($C_{(last)}$) and the elimination rate constant by taking the formula $C_{(last)}/\lambda_z$. The terminal half-life ($T_{½\lambda}$) was calculated by dividing the natural logarithm of 2 by λ_z. Clearance (Cl) was calculated as dose/$AUC_{0-\infty}$. The apparent volume of distribution during the terminal phase (V_z) was calculated as Cl/λ_z. The individual and mean plasma concentration–time curves were evaluated using the non-compartmental method for infusion administration. PK analysis was performed using validated Phoenix WinNonlin Version 6.3 software (Pharsight Corporation, St Louis, MO, USA).

Pharmacodynamic analysis

Concentrations of total ADM (Adrecizumab bound and unbound) were measured using the Spingotest® bio-ADM assay[24]. Mid-regional pro-Adrenomedullin (MR-proADM) was measured using the B·R·A·H·M·S MR-proADM KRYPTOR assay (BRAHMS GmbH, Hennigsdorf, Germany). Endothelin-1 was analysed using an enzyme-linked immunosorbent assay (QuantiGlo®, R&D systems, Minneapolis, MN, USA). Noradrenaline, adrenaline and dopamine levels were measured using routine analysis methods (high-pressure liquid chromatography with fluorometric detection, as

described previously[25]). Plasma renin was analysed using a radioimmunoassay (RENIN III, Cisbio, Codolet, France). In the endotoxemia study, circulating concentrations of the inflammatory cytokines tumour necrosis factor alpha (TNF-α), interleukin (IL) 6, IL-8, IL-10, granulocyte-colony stimulating factor, monocyte chemoattractant protein 1 and interferon gamma-induced protein 10 were determined batchwise using a simultaneous Luminex assay (Milliplex, Millipore, Billerica, MA, USA) according to the manufacturers' instructions.

Endotoxemia-induced symptoms

Subjects scored endotoxemia-induced flu-like symptoms (the 'sickness score') every 30 min throughout the study drug administration day on a numerical response scale ranging from 0 to 10 (0 meaning no complaint at all, 10 extremely severe complaints). LPS-induced flu-like symptoms were not considered to be AEs unless they were of abnormal severity or duration, as judged by the blinded investigators.

Statistical analysis

Data were tested for normality using the Kolmogorov–Smirnov test, and all data were normally distributed. Demographic data were expressed as mean ± standard deviation, and other data were expressed as mean ± standard error of the mean. Differences between placebo and Adrecizumab groups were tested pair-wise using the interaction term from two-way analysis of variance (ANOVA) with repeated measures in the factor time. The dose proportionality of C_{max} and $AUC_{0-\infty}$ of Adrecizumab was assessed using one-way ANOVA followed by a Bonferroni post-hoc test in case a P-value <0.05 indicated non-proportionality. Calculations and statistical analyses were performed using GraphPad Prism version 5 for Windows (Graphpad Software Inc., La Jolla, CA, USA). In case of missing data, values were imputed by the mean of the two adjacent data points. A two-tailed P-value of <0.05 was considered statistically significant.

Results

Study population and subject disposition

A total of 48 subjects were included and randomized in the two studies. The baseline characteristics are listed in **Table 1.** All participating subjects received study medication as intended, completed the study and 90-day follow-up period, and were deemed compliant with the study protocol. No subjects were replaced.

Table 1. Subject characteristics of the first-in-human study (**A**) and the human endotoxemia study (**B**).

A. First-in-human	Placebo	0.5 mg/kg	2 mg/kg	8 mg/kg
	n=6	n=6	n=6	n=6
Age (years)	23 ± 2	23 ± 2	21 ± 1	23 ± 3
BMI (kg/m^2)	25 ± 4	23 ± 2	22 ± 2	23 ± 2
Weight (kg)	80 ± 13	75 ± 7	76 ± 9	76 ± 9
Height (cm)	181 ± 10	182 ± 3	185 ± 3	184 ± 5

B. Endotoxemia	Placebo	0.5 mg/kg	2 mg/kg	8 mg/kg
	n=6	n=6	n=6	n=6
Age (years)	22 ± 1	22 ± 3	23 ± 3	23 ± 3
BMI (kg/m^2)	24 ± 1	23 ± 2	25 ± 2	26 ± 3
Weight (kg)	80 ± 6	76 ± 4	86 ± 8	87 ± 11
Height (cm)	184 ± 5	181 ± 3	187 ± 7	185 ± 9

All presented parameters were determined during the screening visit and are presented as mean ± standard deviation. *Abbreviations: BMI, body mass index.*

Safety and tolerability

Safety and tolerability were the primary endpoints of both studies. Administration of a single dose of Adrecizumab was well tolerated by all subjects in all dose groups, for both studies, and did not result in any safety concerns. All reported AEs were transient, except for one (new-onset type 1 diabetes mellitus, requiring ongoing insulin treatment, detailed below). One serious AE (SAE) was reported in the human endotoxemia study (the aforementioned type 1 diabetes). All variations in laboratory parameters, vital signs and 12- lead ECG were deemed not clinically significant.

<u>First-in-human study</u>

A total of 37 AEs were reported over the 90-day study period (**Table 2A**). Thirty-

four AEs were judged to be mild, whereas three AEs were moderate (these three were deemed 'unrelated' to the study drug) and required treatment: one case of a sexually transmitted disease (Chlamydia infection), one dislocated shoulder and one subject with repeated nose bleeds (details of all AEs are listed in **Table S1A**). Fifteen AEs (41%) were judged by the blinded investigators to be 'unrelated' to the study drug, whereas the rest was deemed 'possibly' related (59%). Twelve AEs were observed in the placebo group, whereas six, 11 and eight AEs were found in the 0.5, 2 and 8 mg/kg Adrecizumab groups, respectively. Commonly reported AEs were nasopharyngitis and headaches, the latter occurring predominantly in the placebo group (see **Table S1A**).

Human endotoxemia study

A total of 27 AEs were observed over the 90-day study period (**Table 2B**). Twenty-five AEs were mild, one was moderate and required treatment (a traumatic carpal bone fracture during the follow-up period; details of all AEs are listed in **Table S1B**) and one was severe and also required treatment (new-onset type 1 diabetes). Eighteen (67%) AEs were judged to be 'unrelated' to the study drug by blinded investigators, and the nine others were deemed to be 'possibly related' (33%). Four AEs were observed in the placebo group, compared with eight, eight and six in the 0.5, 2 and 8 mg/kg Adrecizumab groups, respectively. Commonly reported AEs were nasopharingitis and headaches. One SAE (new-onset type 1 diabetes mellitus) was reported in a subject in the 2.0 mg/kg group. Briefly, this subject experienced weight loss, starting approximately 2 months after study drug administration, and increased thirst and diuresis starting approximately 5 months after study drug administration. Eventually, approximately 8 months after study drug administration, the subject was admitted to the hospital for acute dehydration and a total of 18 kg weight loss. Laboratory results showed hyperglycaemia, ketonuria and elevated glycated haemoglobin, and the subject was diagnosed with type 1 diabetes mellitus. After treatment with insulin and intravenous fluids, he recovered rapidly (<24 h). Currently, no long-term complications have been reported, although the subject remains dependent on exogenous insulin administration. The severity was graded as 'severe'. To investigate the possible relationship with the administration of the study drug, specific type 1 diabetes antibodies were determined on 4 April 2018 in spare baseline samples (taken on 3 January and 6 February 2017, prior to study participation). Causality with Adrecizumab was deemed 'unrelated' because a strong presence of specific type 1 diabetes antibodies was demonstrated in the

baseline blood samples obtained prior to Adrecizumab administration (anti-islet cell antigen, anti-zinc transporter 8, anti-glutamic acid decarboxylase and anti-islet antigen 2 antibodies were all positive at baseline, indicating that the disease was already developing prior to participation in the study, several months before the first clinical signs became apparent).

Table 2. Overview of adverse events in the first-in-human study (**A**) and the human endotoxemia study (**B**).

A. First-in-human	Placebo (n=6)	0.5 mg/kg (n=6)	2 mg/kg (n=6)	8 mg/kg (n=6)	Overall (n=24)
Adverse Events (AE)	12 (32%)	6 (16%)	11 (30%)	8 (22%)	37 (100%)
Severe AE (SAE)	0	0	0	0	0
Discontinued study drug due to (S)AE	0	0	0	0	0
AE of mild intensity	12 (35%)	6 (18%)	8 (23.5%)	8 (24%)	34 (100%)
AE of moderate intensity	0	0	3 (100%)	0	3 (100%)
AE of severe intensity	0	0	0	0	0
Unrelated AE	6 (40%)	3 (20%)	4 (27%)	2 (13%)	15 (100%)
Possibly related AE	6 (27%)	3 (14%)	7 (32%)	6 (27%)	22 (100%)
Probably related AE	0	0	0	0	0
Definitely related AE	0	0	0	0	0

B. Endotoxemia	Placebo (n=6)	0.5 mg/kg (n=6)	2 mg/kg (n=6)	8 mg/kg (n=6)	Overall (n=24)
Adverse Events (AE)	4 (14.8%)	8 (29.6%)	8 (29.6%)	7 (25.9%)	27 (100%)
Severe AE (SAE)	0	0	1 (100%)	0	1 (100%)
Discontinued study drug due to (S)AE	0	0	0	0	0
AE of mild intensity	4 (16%)	8 (32%)	7 (28%)	6 (24%)	25 (100%)
AE of moderate intensity	0	0	0	1 (100%)	1 (100%)
AE of severe intensity	0	0	1 (100%)	0	1 (100%)
Unrelated AE	1 (5.6%)	5 (27.8%)	7 (38.9%)	5 (27.8%)	18 (100%)
Possibly related AE	3 (33%)	3 (33%)	1 (11%)	2 (22%)	9 (100%)
Probably related AE	0	0	0	0	0
Definitely related AE	0	0	0	0	0

Pharmacokinetics

Plasma Adrecizumab concentration-time profiles for both studies are presented in **Figure 2**, and PK parameters are summarized in **Table 3**.

First-in-human study

In all three dose groups, the C_{max} was attained immediately after termination of infusion. Dose-proportional increases in C_{max} and $AUC_{0-\infty}$ were observed

(**Figure S1A**). The $T_{½}$ and the $T_{½}$-dependent parameters of two subjects were excluded from analysis because their elimination phase was not characterized well. The small Cl value (~0.2 ml/h/kg) indicates a slow overall elimination of Adrecizumab from the circulation. A small V_z (~100 ml/kg) indicates that Adrecizumab predominantly remains within the circulation. The terminal elimination $T_{½\lambda}$ of Adrecizumab was ~15 days.

Human endotoxemia study

Similarly to the first-in-human study, values of C_{max} were attained shortly after cessation of study drug infusion. Again, dose-proportional increases in C_{max} were observed (**Figure S1B**). However, dose proportionality was not observed for $AUC_{0-\infty}$ when comparing 0.5 mg/kg with 2.0 mg/kg, and 0.5 mg/kg with 8.0 mg/kg Adrecizumab (**Figure S1B**). PK parameters were virtually identical to those found in the first-in-human study, with the exception of a reduced $T_{½\lambda}$ (a mean of 10 days compared with 14 days; P = 0.02) and V_z (94 ml/kg compared with 78 ml/kg; P = 0.03) in the 0.5 mg/kg dose group.

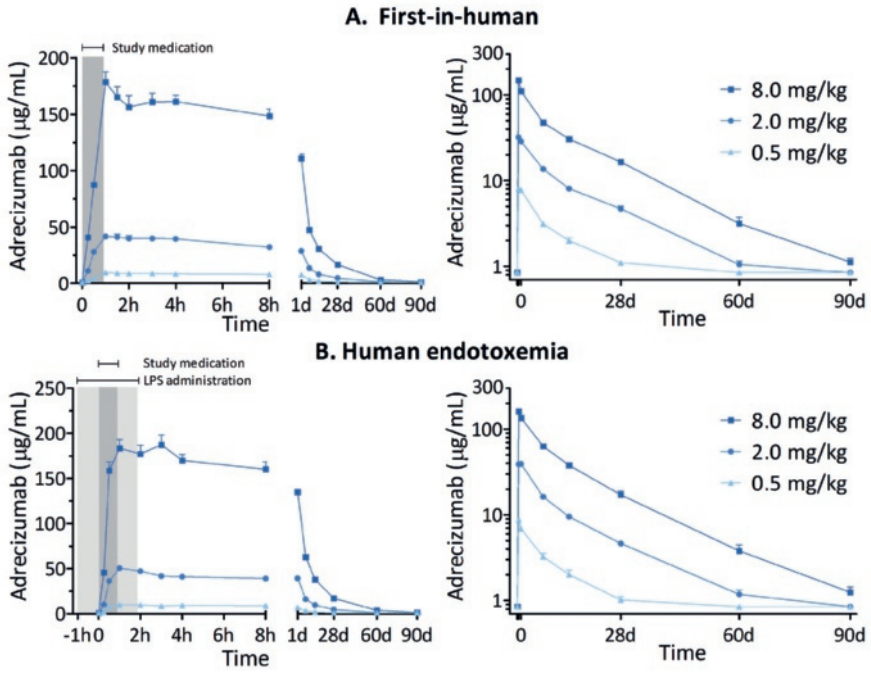

Figure 2. Plasma concentration–time profiles of adrecizumab in the first-in-human study (**A**) and the human endotoxemia study (**B**). Data are expressed as mean ± standard error of the mean. The dark grey area indicates the study drug administration period, and the light grey period the lipopolysaccharide (LPS) infusion period.

Table 3. Pharmacokinetic parameters of Adrecizumab in the first-in-human study (**A**) and the human endotoxemia study (**B**).

A. First-in-human	0.5 mg/kg (n=6)	2 mg/kg (n=6)	8 mg/kg (n=6)
C_{max} (µg/mL)	9.71 ± 0.86	44.1 ± 4.50	179 ± 21.10
$AUC_{0-\infty}$ (µg*h/mL)[#]	2610 ± 222	10700 ± 1870	39000 ± 3340
V_z (mL/kg)[#]	94.4 ± 8.98	95.9 ± 9.70	107 ± 11.50
$T_{1/2}$ (h)[#]	340 ± 6.3	352 ± 51.3	361 ± 49.9
Cl (mL/h/kg)[#]	0.193 ± 0.02	0.193 ± 0.04	0.206 ± 0.02
B. Endotoxemia	**0.5 mg/kg (n=6)[*]**	**2 mg/kg (n=6)[*]**	**8 mg/kg (n=6)**
C_{max} (µg/mL)	10.6 ± 2.07	51.0 ± 6.28	203 ± 17.00
$AUC_{0-\infty}$ (µg*h/mL)	2120 ± 660	12500 ± 1010	46100 ± 5390
V_z (mL/kg)	78.4 ± 11.5	86.9 ± 10.3	93.6 ± 9.9
$T_{1/2}$ (h)	233 ± 85.3	377 ± 67.3	372 ± 46.4
Cl (mL/h/kg)	0.260 ± 0.10	0.162 ± 0.01	0.176 ± 0.02

Data are expressed as mean ± standard deviation.
[a]One subject from the 0.5 mg/kg group was excluded from these variables because the elimination phase was not well characterized.
[b]The $T_{½}$ and the $T_{½}$-dependent parameters for two subjects (one from the 0.5 mg/kg group and one from the 2.0 mg/kg group) were excluded from the mean calculation because their elimination phase was not well characterized.
Abbreviations: $AUC_{0-\infty}$, area under the plasma concentration–time curve from time zero to infinity; C_{max}, highest observed plasma concentration; CL, total clearance calculated; $T_{½\lambda}$, elimination half-life; V_z, volume of distribution characterized by the terminal phase.

Pharmacodynamics

First-in-human study

Administration of adrecizumab did not influence heart rate, mean arterial pressure, peripheral oxygen saturation or temperature (**Figure S2**, all P > 0.05), and there were no significant differences between groups in routine haematological and biochemical safety laboratory measurements (data not shown). Of interest, adrecizumab administration resulted in a rapid and statistically significant dose-dependent increase in total plasma ADM levels (note that the assay detects both bound and unbound ADM), whereas plasma levels of MR-proADM (an inactive peptide originating from the same precursor as ADM) were not increased (**Figure 3A**), implying that the adrecizumab-induced increase in total plasma ADM was not due to increased synthesis. We also measured plasma concentrations of renin, dopamine,

noradrenaline, adrenaline and endothelin-1 in the first-in-human study because these could theoretically counteract possible vasodilatory effects resulting from increased ADM levels. The plasma concentrations of none of these endogenous hormones were influenced relevantly by adrecizumab, as between-group differences were only subtle and not dose dependent (see **Figure S3**).

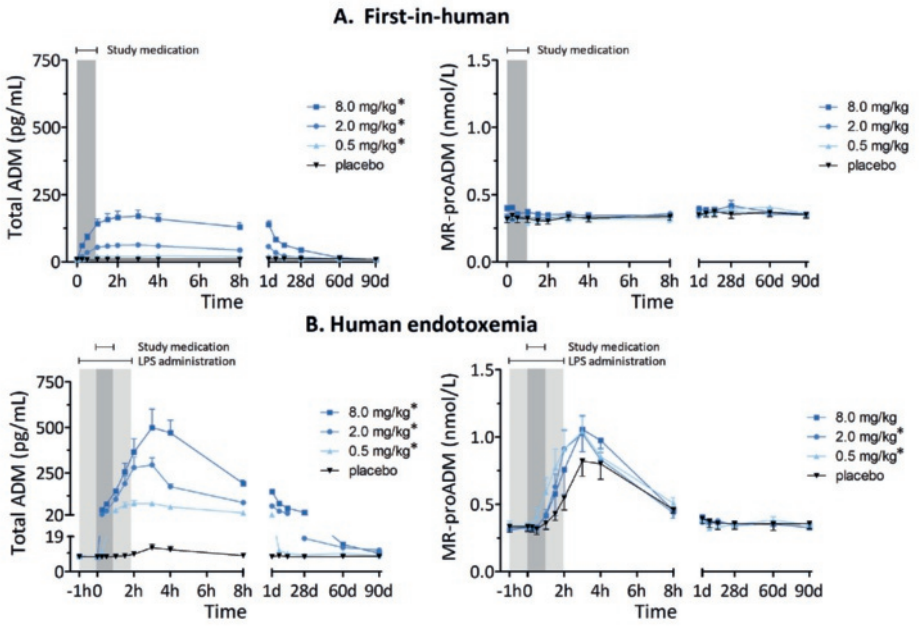

Figure 3. Plasma concentration–time profiles of adrenomedullin (AMD) and mid-regional pro-adrenomedullin (MR-proADM) in the first-in-human study (**A**) and the human endotoxemia study (**B**). Data are expressed as mean ± standard error of the mean. Differences between adrecizumab groups and placebo were evaluated using repeated measures two-way analysis of variance, and interaction term P-values are displayed. * $P < 0.05$.

Human endotoxemia study

LPS administration caused a transient increase in body temperature and heart rate, whereas mean arterial pressure decreased (**Figure 4A-C**). Adrecizumab did not influence the LPS-induced effects on blood pressure or heart rate, although a statistically significant effect was observed for the 0.5 mg/kg dose group for the change in body temperature over time (P = 0.02) (**Figure 4**). However, this difference was very slight, and because no effects for the higher doses were observed, this was likely to be a chance finding. LPS administration caused a transient increase in self-reported flu-like symptoms over time (**Figure 4D**). The highest sickness scores were observed in the placebo group, and resolution of symptoms was significantly more swift in the 8 mg/kg Adrecizumab group compared with placebo (P = 0.01), whereas a trend was observed for the 0.5 mg/kg and 2 mg/kg dose groups (P = 0.08 and P = 0.07, respectively). Similarly to the first-in-human study, Adrecizumab caused a dose-dependent increase in total ADM levels (**Figure 3B**). Compared with the Adrecizumab administration in the absence of LPS, much higher concentrations were reached during systemic inflammatory conditions, which could, at least in part, be explained by the LPS-induced increased synthesis reflected by elevated MR-proADM levels (**Figure 3B**). Expectedly, increased plasma concentrations of various cytokines and chemokines were observed after induction of endotoxemia (**Figure 5**). Typically, cytokine responses showed very high inter-individual variation. For instance, profound between-subject and between-group differences were observed in plasma levels of the archetypal proinflammatory cytokine, and main driver of the inflammatory response, TNF-α, as early as during the first hour after endotoxin administration, when the study drug had not yet been administered. As these differences were already present prior to Adrecizumab or placebo administration, it was not possible to interpret the Adrecizumab-induced effects on absolute cytokine levels. In order to evaluate the effects on cytokine kinetics (e.g. Cl) instead, we normalized cytokine levels to their peak concentrations on a per-subject basis. As depicted in **Figure 5**, Adrecizumab did not influence cytokine kinetics.

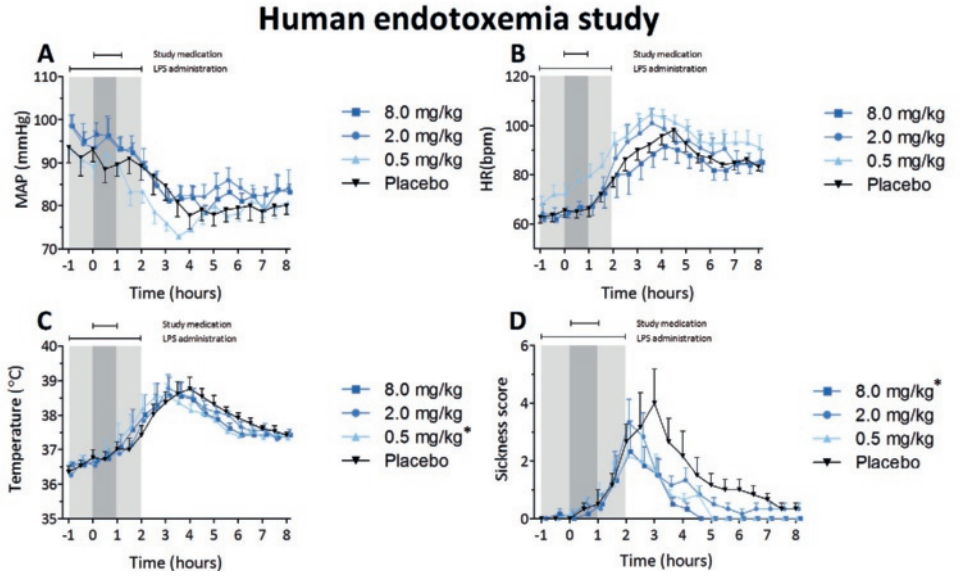

Figure 4. Clinical parameters from the human endotoxemia study: mean arterial pressure (MAP) (**A**), heart rate (**B**), temperature (**C**) and sickness score (**D**). Data are expressed as mean ± standard error of the mean. Differences between Adrecizumab groups and placebo were evaluated using repeated measures two-way analysis of variance, and interaction term P-values are displayed. * P < 0.05
Abbreviations: LPS, lipopolysaccharide.

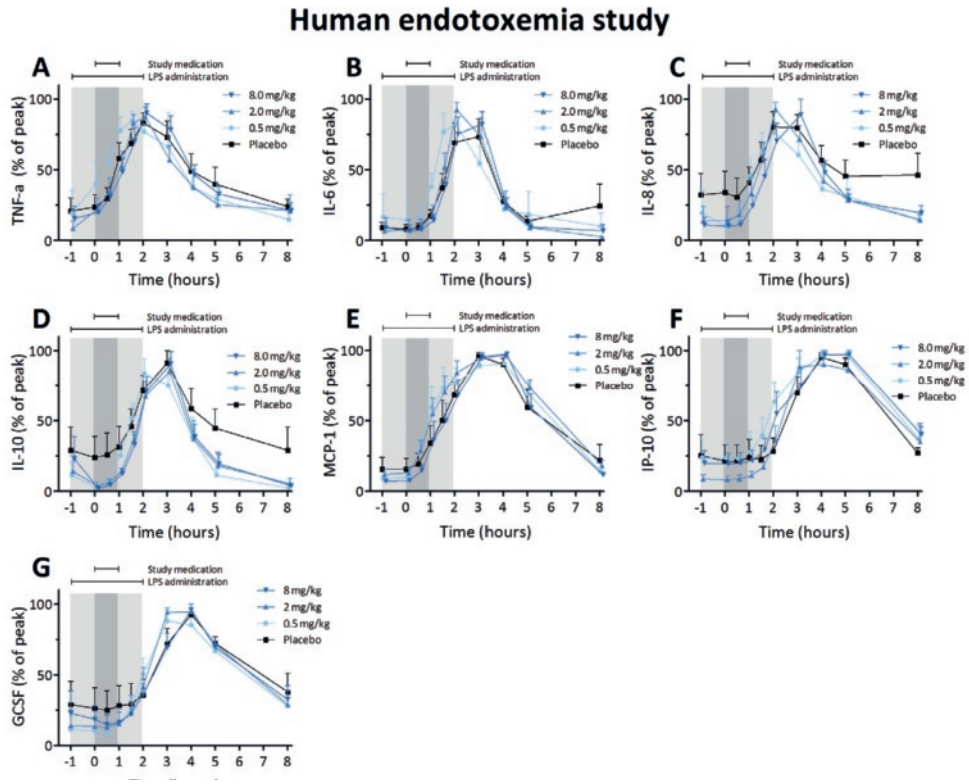

Figure 5. Cytokine clearance from the human endotoxemia study. Data were normalized for the peak cytokine value and are expressed as mean ± standard error of the mean. Differences between Adrecizumab groups and placebo were evaluated using repeated measures two-way analysis of variance, and interaction term P-values are displayed. * $P < 0.05$
Abbreviations: GCSF, granulocyte-colony stimulating factor; IL, interleukin; IP-10, interferon gamma-induced protein 10; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein 1; TNF-α, tumour necrosis factor alpha.

Discussion

In the present study, we evaluated the safety, tolerability, PK and PD of the novel ADM-binding antibody Adrecizumab in a first-in-human study during non-inflammatory conditions, and in a subsequent study during systemic inflammation using the experimental human endotoxemia model. Adrecizumab was well tolerated and demonstrated an excellent safety profile throughout the investigated dose range. Our data revealed dose-proportional maximum concentrations of Adrecizumab which were reached almost immediately after cessation of study drug infusion, a low V_z, low Cl and a long $T_{½\lambda}$. Moreover, Adrecizumab infusion induced a rapid and profound dose-dependent increase in its target peptide ADM, which is thought to explain its mechanism of action[26]. Adrecizumab did not affect vital signs or cytokine clearance, although it did result in a swifter resolution of flu-like symptoms in the human endotoxaemia study.

Safety

One SAE occurred during the 90 day follow-up period, and was demonstrated to be unrelated to the study drug. The majority of AEs were transient, of mild severity and evenly distributed among study groups. The few AEs of moderate severity that occurred (three in the first-in-human study, one in the human endotoxaemia study) were all deemed to be unrelated to the study drug by the blinded investigators. Moreover, we observed no ECG abnormalities, a normal local tolerability at the site of infusion and no clinically relevant changes in vital signs or safety laboratory values. Administration of Adrecizumab during systemic inflammation also did not result in any safety concerns.

Pharmacokinetics

Peak concentrations of adrecizumab were typically observed at the end of infusion and were dose proportional. A low Cl and long $T_{½\lambda}$ of approximately 14 days were observed, which are typical for monoclonal antibodies[27,28]. A low volume of distribution, typical for IgG monoclonal antibodies with a large molecular weight[27,28], indicates that Adrecizumab predominantly remains confined to the blood compartment. Some PK parameters were influenced by systemic inflammation. A slightly lower volume of distribution and $T_{½\lambda}$ were observed for the lowest dose group of 0.5 mg/kg Adrecizumab in the second study, performed under inflammatory conditions. In accordance with these observations, systemic exposure based on $AUC_{0-\infty}$ was slightly lower for the 0.5 mg/kg dose group in the present study. This observation can be

explained neither by nonlinear protein binding of Adrecizumab to its target peptide ADM, nor by increased binding of ADM–Adrecizumab complexes due to inflammation-induced upregulation of the ADM receptors[13,29] because adrecizumab is present in great excess over ADM in the blood. Therefore, these mechanisms cannot relevantly affect Adrecizumab levels. A more plausible explanation is increased clearance of Adrecizumab through inflammation-induced Fc receptor upregulation[27].

Pharamcodynamics

Interestingly, Adrecizumab induced a rapid and profound dose-dependent increase in its target peptide ADM, which is in line with results from animal studies and may be of importance for the underlying mechanism of action. It is well known from various in vitro[11,12,30,31] and in vivo[13,14,32] studies that ADM is a strong endothelial barrier-stabilizing peptide and that administration of ADM to animals with septic shock improves outcome. In fact, the Adrecizumab-induced increase in circulating ADM may well be responsible for the beneficial effects observed in preclinical studies, as a result of the non-neutralizing nature of the antibody allowing ADM to bind with its receptors and activate second messengers[18]. The proposed mechanism of action of Adrecizumab and the role of the observed increase in ADM levels and resulting beneficial effects have recently been put forward[26]. In short, because concentrations of the inactive peptide MR-proADM (derived from the same precursor peptide) were not increased, the Adrecizumab-induced rise in ADM is probably not due to increased production. Hence, it appears plausible that two other mechanisms are responsible for the increase in ADM. First, the binding of ADM to the non-neutralizing antibody Adrecizumab prolongs its $T_{½\lambda}$ because ADM is normally subject to proteolytic degradation by proteases targeting its N-terminus, which is now bound to Adrecizumab[26]. Second, ADM's distribution is shifted towards the blood. This is based upon the fact that ADM can normally diffuse freely across the blood barrier, whereas ADM–Adrecizumab complexes cannot, as Adrecizumab is a large IgG antibody with a low volume of distribution, indicating that it remains confined to the blood compartment. As a consequence, the binding of ADM to the antibody that remains in the circulation will drain ADM from the interstitium.

ADM has been described as a double-edged sword in sepsis[33] because, on the one hand, it improves endothelial barrier stability and outcome in preclinical

animal sepsis models but, on the other hand, it possesses vasodilatory effects which can cause hypotension at high concentrations[16,34,35]. Therefore, it could be hypothesized that the adrecizumab-induced increase in ADM would cause hypotension. However, our data clearly showed that blood pressure was not influenced by Adrecizumab, and that Adrecizumab did not influence the LPS-induced decrease in blood pressure. Moreover, we also measured dopamine and catecholamines in our first-in-human study, to evaluate whether Adrecizumab influenced circulating levels of these vasoactive hormones and therefore might have counteracted, and thereby masked, vasodilation. The finding that the circulating concentrations of these endogenous were not relevantly influenced indicates that the higher levels of (Adrecizumab-bound) ADM do not reach vascular smooth muscle cells and cause vasodilation.

No relevant effects of Adrecizumab on mean arterial pressure, heart rate or inflammatory parameters were observed. In relation to the inflammatory response, it should be stressed that the endotoxemia study was not designed primarily to evaluate this endpoint. Adrecizumab administration was initiated 1 h following the start of the LPS infusion. Therefore, it is not surprising that cytokine kinetics were not influenced by Adrecizumab, as the inflammatory response had already been activated, to a large extent, prior to adrecizumab administration. Unfortunately, it was not possible to evaluate endpoints which were favourably influenced by Adrecizumab in preclinical studies, such as reduced catecholamine demand, organ dysfunction and endothelial barrier leakage[18-20], as the transient and relatively mild human endotoxemia model does not induce such effects. Nevertheless, Adrecizumab did attenuate the sickness score in the highest dose group and tended to decrease it for the two lower doses. This is remarkable, considering the small number of subjects per group and the stringent method of analysis used, yielding limited statistical power to detect significant differences. As no immunomodulatory effects were detected in the present study, it is tempting to speculate that Adrecizumab's effects on the sickness score are mediated through beneficial effects on vascular integrity. As alluded to earlier, our group has previously shown that human endotoxemia does not induce overt microvascular leakage[36] but the analytical methods used may not have been sensitive enough to detect subtler differences in vascular integrity.

Conclusion

Intravenous administration of Adrecizumab was safe and well tolerated in healthy human volunteers, both in non-inflammatory conditions and during systemic inflammation induced by the administration of endotoxin. Moreover, Adrecizumab induced a rapid and profound increase in its target peptide ADM in the blood circulation. This is consistent with previously reported beneficial effects in preclinical sepsis models. Our results pave the way for further investigation of Adrecizumab in septic patients. A phase II proof-of-concept, precision medicine study in septic shock patients has recently started recruitment and is anticipated to enrol 300 patients (clinicaltrials.gov, NCT-number: NCT03085758). Furthermore, as endothelial dysfunction is not restricted to sepsis/septic shock but also occurs in other diseases[37-39], Adrecizumab might have broader applicability.

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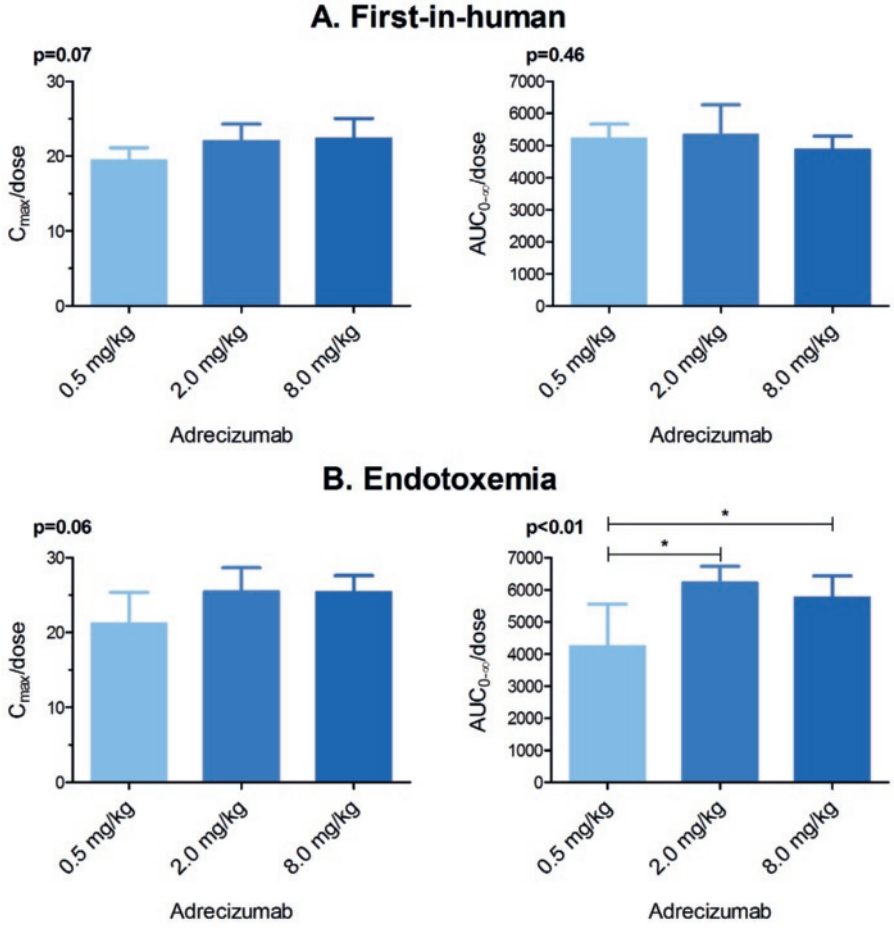

Figure S1. The dose proportionality of Cmax and $AUC_{0-\infty}$ of adrecizumab was assessed using one-way analysis of variance followed by a Bonferroni post-hoc test in case a P-value < 0.05 indicated nonproportionality. Data from both the first-in-human study (**A**) and the human endotoxaemia study (**B**) were analysed. Data are expressed as mean ± standard deviation. *Abbreviations: $AUC_{0-\infty}$, area under the plasma concentration–time curve from time zero to infinity; C_{max}, highest observed plasma concentration.*

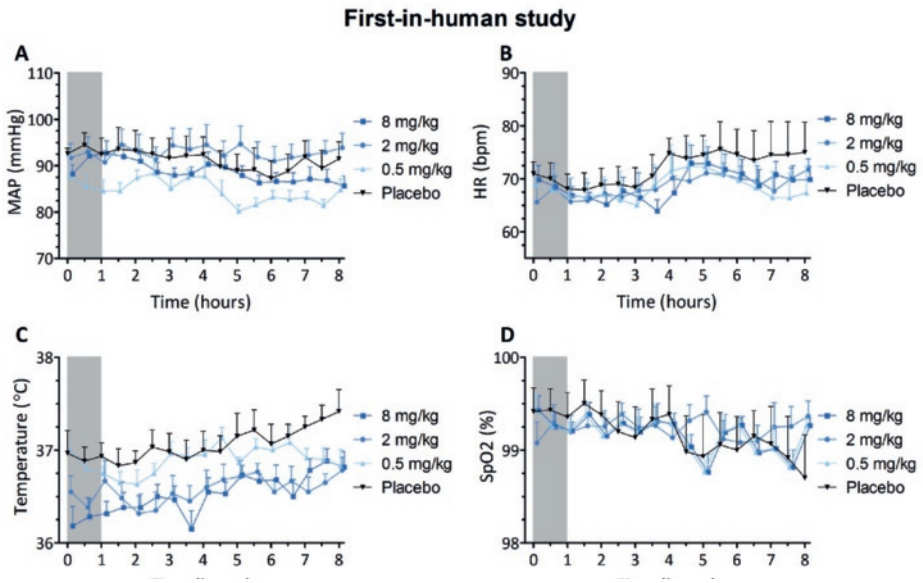

Figure S2. Vital signs in the first-in-human study: mean arterial pressure (**A**), heart rate (**B**), temperature (**C**) and peripheral oxygen saturation (**D**). Data are expressed as mean ± standard error of the mean. The dark grey area indicates the study drug administration period. Differences between Adrecizumab groups and placebo were evaluated using repeated measures two-way analysis of variance, and interaction term P-values are displayed. *P < 0.05.

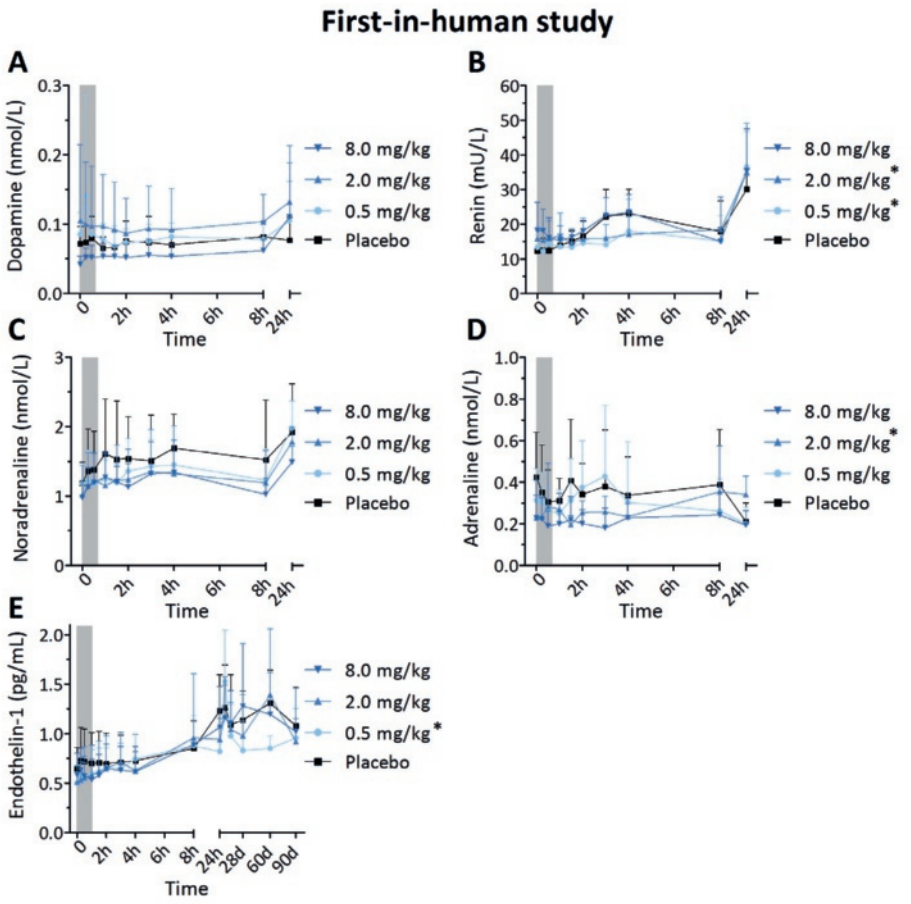

Figure S3. Plasma concentrations of dopamine (**A**), renin (**B**), noradrenaline (**C**), adrenaline (**D**) and endothelin-1 (**E**), measured in the first-in-human study. Data are presented as mean ± standard error of the mean. Differences between adrecizumab groups and placebo were evaluated using repeated measures two-way analysis of variance, and interaction term P-values are displayed. *P < 0.05.

Table S1. Summary of adverse events by system organ class for the first-in-human study (**A**) and the human endotoxemia study (**B**).

A. First-in-human	Placebo (n=6)	0.5 mg/kg (n=6)	2 mg/kg (n=6)	8 mg/kg (n=6)	Overall
General disorders					
Nasopharingitis	3	4	3	3	13
Nervous system					
Headache	6	1	0	2	9
Cardiovascular					
Lightheadedness during exercise	0	0	1	0	1
Gastrointestinal					
Abdominal pain	0	0	1	0	1
Travelers diarrhea	0	0	1	0	1
Infectious					
STD (Chlamydia)	0	0	1	0	1
Skin infection (wound ankle)	1	0	0	0	1
Musculoskeletal					
Dislocated shoulder	0	0	1	0	1
Back pain	0	1	0	0	1
Chest pain	1	0	0	0	1
Sensation of light pressure on shoulder	0	0	1	0	1
Muscle soreness	0	0	0	1	1
Thumb pain	0	0	0	1	1
Dermatological					
Erythemateus rash at site of infusion	1	0	0	0	1
Eczema				1	1
Peri-anal fungal skin infection with eczema-like symptoms	0	0	1	0	1
Other					
Repeated nose bleeds	0	0	1	0	1

Table S1. continued.

B. Human endotoxemia†	Placebo (n=6)	0.5 mg/kg (n=6)	2 mg/kg (n=6)	8 mg/kg (n=6)	Overall
General disorders					
Nasopharingitis	2	5	4	1	12
Nervous system					
Headache	1	1	1	0	3
Twitches in left arm	0	1	0	0	1
Paresthesia	0	0	1	0	1
Respiratory					
Apnea and oxygen saturation while sleeping	0	0	0	1	1
Gastrointestinal					
Rectal blood loss	0	1	0	0	1
Infectious					
Cold sore (upper lip)	0	0	0	1	1
Musculoskeletal					
Bone fracture	0	0	0	1	1
Back pain	0	0	1	0	1
Dermatological					
Erythemateus rash at site of infusion	0	0	0	1	1
Metabolic					
New onset type I diabetes	0	0	1	0	1
Other					
Nose bleed	1	0	0	1	2
Swelling upper eye lid	0	0	0	1	1

† LPS-induced symptoms were not included as AEs unless they were of abnormal intensity or duration, as decided by the blind investigator.

Chapter 13

Study protocol: A double-blind, placebo-controlled, randomised, multicentre, proof-of-concept and dose-finding phase II clinical trial to investigate the safety, tolerability and efficacy of Adrecizumab in patients with septic shock and elevated adrenomedullin (AdrenOSS-2)

Christopher Geven, Alice Blet, Matthijs Kox, Oliver Hartmann, Paul Scigalla, Jens Zimmermann, Gernot Marx, Pierre-François Laterre, Alexandre Mebazaa and Peter Pickkers

British Medical Journal Open 2018; December 31, *in press*

Abstract

Introduction

Sepsis remains a major health problem with an increasing incidence, high morbidity and high mortality. Apart from treatment with antibiotics and organ support, no approved specific adjunct therapies currently exist. Adrenomedullin (ADM) is a vasoactive peptide. High plasma concentrations of ADM correlate with worse outcome in sepsis patients. Preclinical work with the non-neutralizing ADM-binding antibody Adrecizumab showed promising effects in animal models of septic shock, including improved vascular barrier function, reduced vasopressor demand and organ dysfunction and increased survival. Therapeutic use of Adrecizumab may therefore improve outcome in critically ill patients with septic shock and high ADM plasma concentrations. Phase I studies in healthy volunteers did not reveal any safety concerns. In this biomarker-guided trial, the safety and efficacy of Adrecizumab will be investigated in patients with septic shock.

Methods and analysis

We describe a phase II, randomised, double-blind, placebo-controlled, biomarker-guided, proof of concept and dose-finding clinical trial in patients with early septic shock and high concentration of circulating ADM. A total of 300 patients will be enrolled at approx. 30 sites within the European Union. Patients are randomized to receive active treatment (2 and 4 mg/kg Adrecizumab) or placebo, in a 1:1:2 ratio. Patient selection is not only guided by clinical parameters, but also biomarker-guided by measurement of circulating biologically active ADM concentration at admission. Primary endpoint is safety and tolerability of Adrecizumab over a 90 day period. A key secondary endpoint is the Sepsis Severity Index (SSI) over a 14-day period.

Ethics and dissemination

This study is approved by relevant institutional review boards/independent ethics committees and is conducted in accordance with the ethical principles of the Declaration of Helsinki, the European Medicines Agency guidelines of Good Clinical Practice, and all other applicable regulations. Results of this study will be published in a peer-reviewed scientific journal.

ClinicalTrial.gov registration number

NCT03085758

Introduction

Worldwide, sepsis is a major health problem, with an increasing incidence and high mortality[1-3]. It is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection[4]. Septic shock is defined as a subset of sepsis in which profound circulatory, cellular, and metabolic abnormalities occur, which are associated with an increased risk of mortality[4]. The most prominent abnormalities are vasodilation and loss of vascular integrity, resulting in hypotension, and ultimately, in organ dysfunction and death[5]. Besides antibiotics and organ supportive therapies such as vasopressors, mechanical ventilation and renal replacement therapy (RRT), there are currently no sepsis-specific adjunctive therapies registered.

Adrenomedullin (ADM) is a vasoactive peptide hormone that plays an important role in sepsis. Circulating ADM exerts endothelial barrier-stabilizing effects and maintains vascular integrity[6-10]. ADM has vasodilatory properties in the vascular interstitium, and at high concentrations, as observed during sepsis, may contribute to hypotension[11-13]. Elevated concentrations of plasma ADM at admission have been reported in septic patients, and these were correlated with vasopressor requirement, organ dysfunction and mortality[14-16]. The cut-off value of biologically active ADM (bio-ADM) of 70 pg/mL at admission was found to predict mortality for sepsis patients[14]. This cut-off has been validated in independent, large multicentre studies[15,17,18].

Based on these data, ADM may be an interesting therapeutic target for sepsis. A potential new adjunctive therapy for the treatment of septic shock is Adrecizumab (previously known as HAM8101). It is a *non-neutralizing* ADM-binding antibody that has shown beneficial effects in preclinical studies. Adrecizumab reduced vascular leakage, organ dysfunction and need for vasopressor treatment during cecal ligation and puncture (CLP) induced sepsis in several animal studies, and improved urine output and survival[19-21]. Importantly, Adrecizumab administration was not associated with any safety concerns in the first-in-human phase I study in healthy volunteers (n=24)[22,24] and in a follow-up study in healthy volunteers, which were intravenously challenged with lipopolysaccharide (LPS) to induce systemic inflammation (also n=24)[23,24]. Of note, in the latter study, LPS-induced flu-like symptoms resolved more swiftly in Adrecizumab-treated subjects compared to the placebo-treated subjects. Pharmacokinetic (PK) analysis of Adrecizumab showed a half-life of approximately 14 days, indicating that administration

of a single dose is sufficient to achieve excess of plasma concentrations of the antibody over adrenomedullin for the entire sepsis period.

Based on these preclinical and human phase I data, it is hypothesised that therapeutic use of Adrecizumab may improve endothelial dysfunction, restore and maintain vascular integrity and augment hemodynamics in critically ill patients with sepsis and septic shock. In the trial described in the present work, the safety, tolerability and efficacy of Adrecizumab is investigated in patients with early septic shock and elevated concentrations of circulating bio-ADM. This will be one of the first precision medicine, biomarker-guided studies in septic patients.

Methods and analysis

Design and setting

AdrenOSS-2 is a phase II, randomised, double-blind, placebo-controlled, biomarker-guided, proof-of-concept and dose-finding clinical trial that is currently being conducted in patients with early septic shock and elevated concentration of circulating bio-ADM (> 70 pg/mL). A total of 300 patients will be recruited in medical, surgical and mixed Intensive Care Units (ICU) at approximately 30 sites across Belgium, France, Germany, the Netherlands and Italy (see clinicaltrials.gov for a list of current centres). Patient selection is guided by clinical parameters as well as by biomarker concentrations, by measuring circulating bio-ADM (sphingotest® bio-ADM, sphingotec GmbH, Hennigsdorf, Germany)[25]. Based upon preclinical studies, two dosages of Adrecizumab will be investigated (2 and 4 mg/kg bodyweight), in addition to a placebo control arm. After informed consent has been signed by the patient or his/her legal representative, circulating bio-ADM concentrations will be assessed. If bio-ADM concentrations are > 70 pg/mL, the clinical coordination center (CCC) will be contacted for final confirmation of patient eligibility and the patient will be randomized. An interim analysis for futility is planned after 150 patients have completed day 28 of the study. An overview of the study design is depicted in **Figure 1** and study procedures in **Figure 2**.

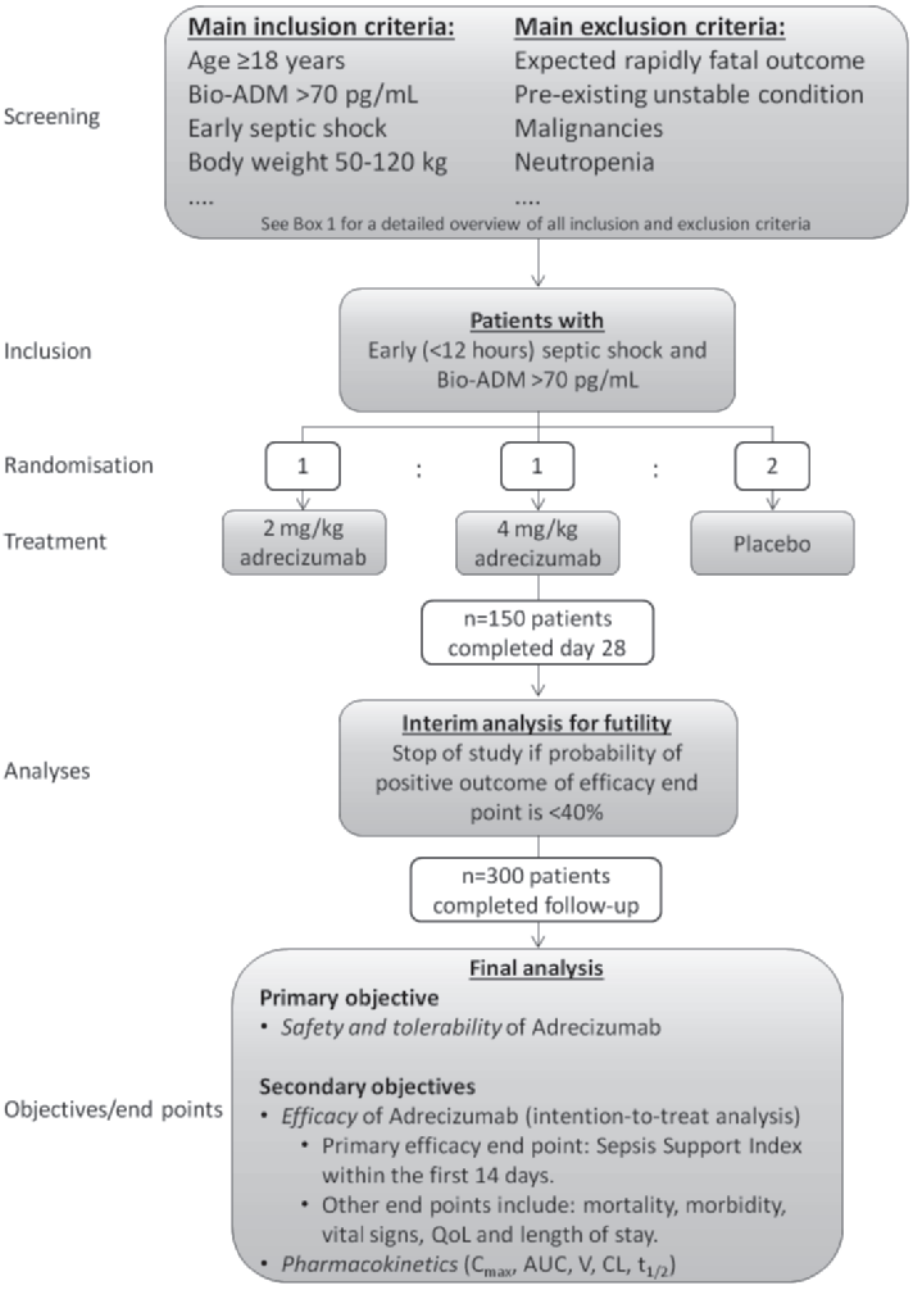

Figure 1. Study design. *Abbreviations: ADM, adrenomedullin; AUC, area under the curve; CL, systemic clearance; QoL, quality of life.*

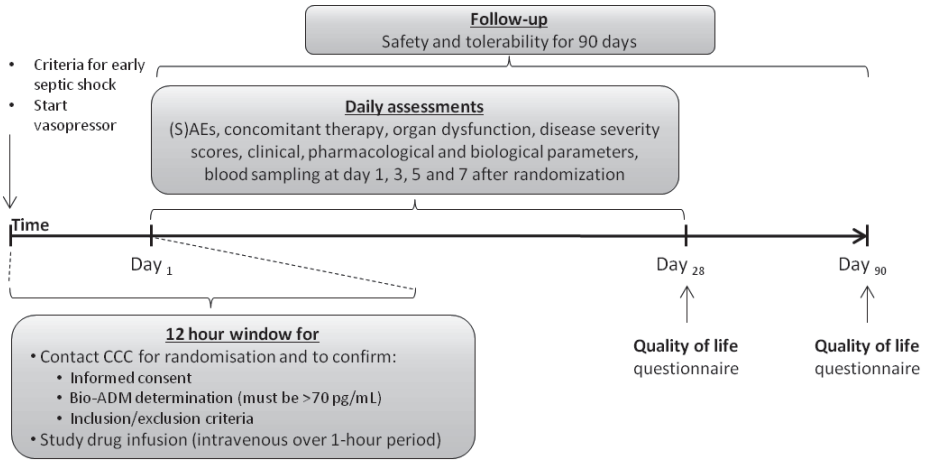

Figure 2. Study timeline. *Abbreviations: ADM, adrenomedullin; CCC, clinical coordination centre; SAE, severe adverse event.*

Primary objective

The primary objective is safety and tolerability, consisting of: mortality possibly related to Adrecizumab, interruption of infusion due to suspected intolerability of Adrecizumab, new treatment-emergent adverse events (AEs) possibly related to Adrecizumab and changes in severity and frequency of treatment-emergent AEs. During the study, an independent Data and Safety Monitoring Board (DSMB) will review safety data on at least a monthly base.

Secondary objectives

The secondary objectives are related to the efficacy and PK of Adrecizumab. The primary efficacy endpoint, the 'Sepsis Support Index' (SSI), is a composite endpoint reflecting organ dysfunction or death within the first 14 days of follow-up. More precisely: within the first 14 days of follow-up, every day on which a vasopressor or mechanical ventilation is used, or renal dysfunction (defined as renal Sequential Organ Failure Assessment [SOFA] score=4) is apparent, or the patient is not alive anymore, is counted as l. The sum over the 14 day follow-up period is defined as the SSI score, which can have a maximum of 14 and a minimum of 1 (as vasopressor usage on day 1 is an inclusion criteria). The calculation of the SSI is further illustrated in **Figure 3**. Additional secondary objectives include: SSI at day 28 of follow-up, penalised SSI (patients who die get penalised with the maximum score), individual SSI components, persistent organ dysfunction or death at day 14 and 28 of follow-up[26], day 28 and day 90 mortality rate and quality of life (Euro-

QOL-5), change over time in SOFA and other parameters such as functional parameters (including, but not limited to heart rate, blood pressure, PaO_2/FiO_2, fluid balance, blood lactate, creatinine, pro-enkephalin, mid-regional-proADM, inflammatory markers, including procalcitonin and interleukin-6), total duration of vasopressor/catecholamine use as well as length of stay at ICU/hospital.

For the PK substudy (n=80 patients), endpoints are key PK parameters, including peak plasma concentrations (C_{max}), systemic exposure (area under the curve), volume of distribution (V), systemic clearance (CL) and elimination half-life ($t_{1/2}$) of Adrecizumab.

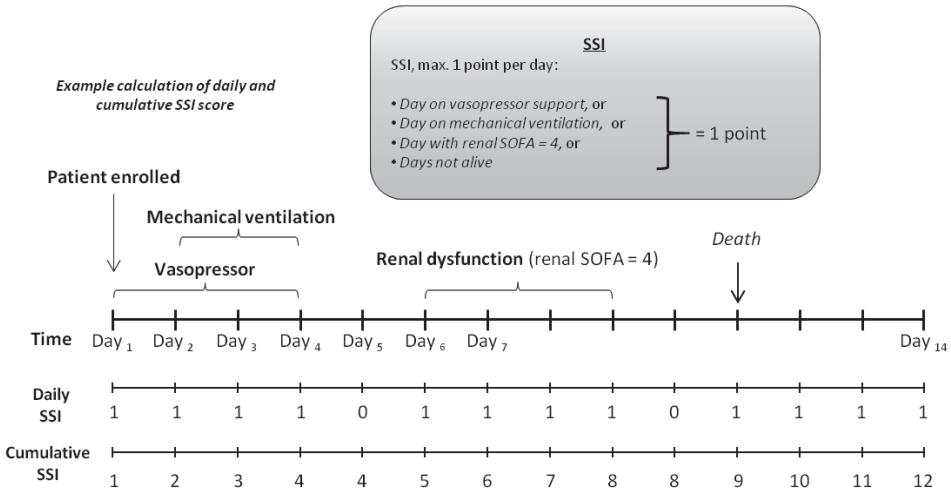

Figure 3. Primary efficacy endpoints: 14-day Sepsis Support Index (SSI) example calculation.

Patient selection

A total of 300 adult patients with early septic shock and elevated bio-ADM concentration will be randomised. Early septic shock is defined as sepsis with hypotension (mean arterial pressure <65 mmHg) refractory to fluid rescucitation and requiring vasopressor therapy[4]. Patients with a measurement of circulating bio-ADM >70 pg/mL will be eligible to be randomised. The cut-off point for bio-ADM of 70 pg/mL was selected based on the specific needs and purpose of this study. Per patient data available for this evaluation included data from ALBIOS, Frog-ICU and AdrenOSS-1 studies, to name the largest and most relevant, as well as data from healthy normal individuals. Specific needs to be met for the study were that patients with normal bio-

ADM, as well as low severity and low expected mortality were to be excluded, to maximise the observable treatment effect, while keeping the eligible population as large as possible. The window for inclusion and infusion of study medication is 12 h following initiation of vasopressor therapy. A lactate concentration <2 mmol/L is not an inclusion criteria, as concentrations may change quickly in response to initial therapy. Patients will be screened for clinical inclusion and exclusion criteria (**Table 1**). Screening and enrolment logs will be maintained for all patients. For patients not enrolled in the study, the reason for non-enrolment is documented. Patients will undergo various screening assessments, including recording of information on hospital and ICU admission (date, time, location before admission, diagnosis, origin of sepsis), documenting of relevant ongoing conditions, relevant medical history and comorbidities present or treated within the last year (cardiovascular and non-cardiovascular), concomitant medication use, age, gender, ethnic origin, physical examination including weight and height, blood sampling for laboratory examinations and bio-ADM measurement, pregnancy test (urine or serum), recording of 12-lead electrocardiography (ECG), and calculation of APACHE (Applied Physiology And Chronic Health Evaluation) II and SOFA score. Eligibility will be confirmed by the clinical coordination centre (CCC) in Brussels, Belgium. Patients that fulfill all inclusion criteria and none of the exclusion criteria will be eligible to be randomised.

Measuring bio-ADM

For measurement of bio-ADM, 5 mL ethylenediaminetetraacetic acid (EDTA) blood will be collected after written informed consent is obtained. After centrifugation (2500 G, 15 min, 20 °C), bio-ADM levels are determined using a fully validated, CE-marked, commercially available immunoluminometric assay (sphingotest bio-ADM assay). This assay is performed locally by trained personnel. The assay is highly specific for C-terminally amidated ADM (bio-ADM). Each patient sample will be measured in duplicate, and in parallel 2 calibrators (1 with a concentration around the decision-making point [70 pg/mL]) will be run in triplicate along with each patient sample. The functionality of the measuring system will be checked on a monthly basis at each site. Finally, bio-ADM will be remeasured from banked aliquots in batch at a central laboratory to verify locally gained results. Further details about the assay are described elsewhere[25].

Table 1. Inclusion and exclusion criteria

Inclusion criteria	
1.	Written informed consent by patient or legal representative (according to country-specific regulations)
2.	Male and female patients, age ≥18 years.
3.	Body weight 50–120 kg.
4.	Bio-ADM concentration >70 pg/mL.
5.	Patient with early septic shock (start of vasopressor therapy <12 hours).
6.	Women of childbearing potential must have a negative serum or urine pregnancy test before randomization and have to use a highly effective method of contraception.
Exclusion criteria	
1.	Moribund.
2.	Pre-existing unstable condition (e.g., a recent cerebral hemorrhage or infarct, a recent acute unstable myocardial infarction (all <3 months), congestive heart failure NYHA class IV.
3.	Patients who required cardiopulmonary resuscitation in the last 4 weeks prior to evaluation of enrolment.
4.	Severe chronic obstructive pulmonary disease (COPD) with chronic oxygen need at home (GOLD IV).
5.	Any organ or bone marrow transplant in the last 4 weeks prior to evaluation of enrolment.
6.	Uncontrolled serious hemorrhage (≥2 units of blood / platelets in the previous 24 hrs). Patients may be considered for enrollment if bleeding has stopped and patient is otherwise qualified.
7.	Uncontrolled hematological / oncological malignancies.
8.	Absolute neutropenia <500 per µL.
9.	Severe chronic liver disease (Child-Pugh C).
10.	Systemic fungal infection or active tuberculosis.
11.	Neuromuscular disorders that impact breathing/spontaneous ventilation.
12.	Burns >30% of body surface.
13.	Plasmapheresis.
14.	Women who are pregnant or nursing.
15.	Participation in a clinical trial involving another investigational drug within 4 weeks prior to inclusion.
16.	Unwilling or unable to be fully evaluated for all follow-up visits.

Randomisation
Patients are randomly assigned to receive active treatment (2 and 4 mg/kg Adrecizumab) or placebo, using a block randomisation scheme (1:1:2 treatment allocation ratio). A randomisation code list will be generated by an independent statistician not involved in the study. For each centre, study medication is provided in boxes containing 4 pairs of vials according to the 4-block randomisation list, allowing stratification by centre.

Informed consent
Prior to any study-related procedures, patients must provide informed consent in accordance with the EU Clinical Trial Directive, the Declaration of Helsinki and ICH-GCP requirements. Informed consent is obtained according to local requirements in participating countries. Written informed consent is obtained by trained investigators after providing adequate verbal and written information about the study (in order to fully understand the study and any risks it entails), and giving the patient opportunity to ask questions as well as appropriate time to decide on participation in the study. For patients unable to provide consent themselves due to their medical condition, written informed consent is to be obtained by the patient's legal representative or by other accepted procedures according to applicable national law and local regulations, for example, consent by relatives or family members. In addition, retrospective patient consent to voluntary continue the study will be obtained once the patient has sufficiently recovered. Patient and/or the patient's legal representative can withdraw their consent on study participation at any time without providing an explanation.

Blinding
The study will be performed in a double-blinded fashion. All study personnel, including the investigator and site staff, monitors, sponsor and Contract Research Organisation (CRO) staff will be blinded to treatment assignment until study closure. The randomisation list is kept strictly confidential by the data management vendor and accessible only to authorised perosns who are not involved in the conduct of the study. In case of emergency, blinding will only be broken if specific emergency treatment would be indicated by knowing the treatment status of the patient. Specific emergency envelopes will be available at each site. The investigator is required to notify the sponsor within 24 hours following the code break reporting the reason for unblinding. The investigational drug and its matching placebo are indistinguishable and all

study drug kits will be packed in the same way. Unblinding will be authorised by the sponsor after completion of the study, locking of the database and performance of a blinded data review.

Study intervention
A single dose of the study drug (2 or 4 mg/kg Adrecizumab, or placebo) is administered over a 1-hour period by continuous intravenous infusion, as soon as possible, but at the latest, within 12 hours following start of vasopressor therapy. Study drug is administered separately from any concomitant drugs using a dedicated lumen of a central venous catheter or a separate peripheral line. Study medication is provided in boxes according to the 4-block randomisation list. Each box contains 4 pairs of vials for a 1:1:2 treatment allocation ratio. The following pairs of vials are supplied in the box, in a blinded fashion: a set of 2 vials of Adrecizumab (for reconstitution of the 4 mg/kg dose), a set of 1 vial of Adrecizumab and 1 vial of placebo (for reconstitution of the 2 mg/kg dose) and 2 sets of 2 placebo vials. All vials are indistinguishable from each other, containing the same volume of solution, the same aqueous buffer and identical packaging. The study drug, adjusted to the patient's body weight, has to be reconstituted from a pair of vials. All study drug are stored in a secure and adequately temperature-monitored pharmacy storage facility at 2-8 °C.

Concomitant medication
There are no specific restrictions regarding use of concomitant medication or other therapies. All patients will be treated according to 'International Guidelines for Management of Severe Sepsis and Septic Shock'[27]. All concomitant medical treatments and medication will be recorded from inclusion until day 28 or ICU discharge (whichever comes first).

Patient and public involvement
Patients and the public were not involved in elaboration of the study protocol. There is no plan to disseminate the results directly to the study participants. Results will be published in a peer-reviewed journal and presented on conferences.

Statistical and analytical plan

Sample size calculations

The sample size was calculated for the primary efficacy endpoint (SSI up to day 14). A sample size of n=150 patients is planned for the combined treatment groups receiving 2 and 4 mg/kg Adrecizumab. As both dosages result in an excess of antibody over the target peptide ADM, no difference in treatment effect is expected between the dosage groups. Therefore, the two dosage groups are pooled together for the final analysis, unless either dose is insufficient or safety and tolerability analysis indicate that one dose is not safe or tolerable. Power calculation was based on simulation analyses. The distribution of the SSI was based on real patient data from the ALBIOS study (n=539)[15] and underlying assumptions were re-evaluated using results from the AdrenOSS-1 observational study[18]. Based on the AdrenOSS-1 study performed in septic patients, we anticipate a median SSI in the control group of 4 [IQR 2-11], while in the ALBIOS study the median was 7 [IQR 4-14] (these medians reflect a selection of patients with septic shock and bio-ADM >70 pg/mL). However, due to the non-normal distribution of the SSI, the median is still highly volatile (the majority of patients have either a low SSI [1-3 days, if improving and discharged early], or a high SSI [14 days, as patients that die within the first 14 days are usually on organ support while alive and in ICU]). For the simulations, a sample size of n=150 per group (treatment or placebo), and an effect size resulting in approximately 10% decrease in SSI in the Adrecizumab-treatment group (compared with the simulated control group) resulted in a power of the study of >80% to demonstrate an improvement of SSI of >0 with at least 80% probability. The 80% probability corresponds to the lower limit of the 60% confidence interval of the effect estimate, delta SSI, which is based on the estimated difference of location from the Wilcoxon test. If the simulated lower limit of delta SSI was >0, the simulation run reached the endpoint.

Statistical analyses

Continuous variables will be summarised by the number of patients, mean, SD or median, quartile and range, as appropriate. Categorical variables will be summarized using number and percentage by category. Demographic and medical background data, secondary endpoints and safety variables will be analysed by means of descriptive and exploratory methods. Regarding the primary endpoint (safety), all AEs will be listed. The number and percentage of patients experiencing one or more AEs will be summarised by treatment

arm/control group, relationship to study drug and severity/grade. Severe Adverse Event (SAE)-specific listings for each patient population will be generated on reported SAEs, but not as SUSARs. The same will be made for treatment-related SAEs. Mortality analysis is described below.
The primary analysis for efficacy will be performed as an intention-to-treat analysis based on the combined dosage groups of Adrecizumab (n=150 patients total) versus placebo. A secondary analysis will compare the two doses for differences in efficacy. In case patients did not receive the treatment they were randomised to, an analysis based on the actual treatment will also be performed (as-treated analysis). The primary efficacy endpoint, 14-day SSI, will be analysed using the non-parametric Wilcoxon test, to estimate the treatment effects (based on the Wilcoxon estimate for difference in location) as well as its confidence interval. First, it will be determined whether the improvement in SSI due to treatment is >0 with at least 80% probability (based on the lower limit of the one-sided confidence interval of the effect estimate of the Wilcoxon test). If this is achieved, the classical p-value from the Wilcoxon test will also be calculated. All-cause mortality will be evaluated using Kaplan-Meier plots comparing treatment (separate for each dose, as well as a comparison combining both doses into one group) versus placebo (log-rank test) and Cox regression modelling including covariates to adjust for potential confounders. Potential confounders include age, gender, mean arterial pressure, heart rate, source of infection, blood culture, comorbidities and initial SOFA score as well as variables showing significant between-group differences (despite randomisation). In order to identify subgroups which may possibly benefit more from Adrecizumab treatment, interactions with other drugs, as well as exploratory subgroup analyses are planned in patients, defined by disease severity, biomarkers, concomitant medication or other clinical data. The subgroup analyses is nevertheless purely exploratory. Subgroups will be defined by tertiles for continuous variables. For categorical variables, categories will be summarised such that they best represent tertiles if more than three categories are available. Statistical analysis of secondary endpoints is exploratory, and will be specified ina separate statistical analysis plan, which is to be finished before conclusion of the study.

Interim analysis with futility stop
An unblinded interim analysis is planned after 50% of patients completed the study on day 28. The study will be terminated if the probability of a positive outcome after recruitment of all patients is below 40%, based on the

primary efficacy endpoint 14 day SSI. In case the futility stop is reached, but if some of the other efficacy endpoints show a promising outcome for the full study, the futility stop may be suspended. Statistical consequence of applying the futility analysis was included in the power simulation. An independent statistician is responsible for analysing the data at interim analysis, and the steering committee, as well as the sponsor, will remain blinded until the end of the study. Note that the interim analysis focuses on futility only, potential termination of the trial based on harm is based on the reviewing and evaluation of unblinded data on safety and mortality by the DSMB (described further below).

Data quality assurance

All data management activities are done according to ICH-GCP as required by regulatory agencies. A commercial CRO, M.A.R.C.O. GmbH & Co. KG (M.A.R.C.O®), will be responsible for data management. All sites will maintain source documentation and enter patient data into an electronic case report form (eCRF). The clinical center is responsible for the secure and restrictive archiving of source data for at least 15 years or until the written notification from the sponsor that the documents are no longer required. During the required period, the clinical center will ensure that archived data and documents will be undamaged, legible and accessible to the sponsor and/or for regulatory purposes, if required. The study master file, the ECRFs, code envelopes and other material supplied for the performance of the study will be retained by the sponsor according to applicable regulations and laws, including the new GDPR (see also the section on 'Confidentiality'). Regarding the eCRF, automated and manual checks will be performed to ensure completeness and consistency of the data, and investigator site personnel seeking access must go through training processes before access to the system is granted. The eCRF was designed by M.A.R.C.O.® in the Amedon system. Validation checks are implemented in the system or programmed with SAS®, version 9.1 or higher, according to the data validation plan set up by M.A.R.C.O.®.

Safety assessments

Medication error

Adequately trained hospital staff will prepare, double-check and administer study medication. The dose levels that are administered in the study have not caused any safety concerns in previous studies in healthy volunteers[22-24] or in preclinical safety and toxicological studies in animals and non-human

primates. The risk for adverse health effects due to medication errors are thought to be minimal.

Overdose risks

No drug-specific antidote for Adrecizumab is available. An overdose is defined as any dose higher than the assigned treatment dose. However, if by accident, the maximum volume would be withdrawn from a pair of Adrecizumab vials during preparation of study medication, this would not exceed the tested maximum dose of 8 mg/kg Adrecizumab in healthy volunteers, which did not result in any safety concerns[22-24].

AE reporting

All patients are monitored for adverse events (AEs). AEs are defined as any untoward medical occurrence in a patient administered a product and which does not necessarily have a causal relationship with this treatment. Investigators must document all AEs (whether serious or non-serious and judged related or unrelated to the study drug) that occur during the study period extending from day 1 (inclusion) until 90 days after study drug administration in the eCRF. If the AE is serious, a 'serious adverse event report form' must also be sent to the safety contact of the sponsor (spm^2, Safety Projects & more GmbH, Hirschberg an der Bergstraße, Germany) within 24 hours of becoming aware of the SAE. The severity of the AE will be rated as 'mild', 'moderate', 'severe', 'life-threatening', 'disabling' or 'death related to event'. Investigators will use medical judgement to determine whether there is evidence for a causal relationship and will describe this causality using terms such as 'certain', 'probably/likely', 'possible', 'unlikely' or 'unrelated'. All AEs will be followed-up until they have abated, or until a stable situation has been reached, and will be reported as such.

External data monitoring committee

An independent DSMB has been established to monthly review safety data including SAEs and, overall safety data, and will judge the relevance of events for patient safety. DSMB members will have no direct relationship to the study or to the study sponsor. The DSMB, composed by two clinical experts in the field of sepsis, a biostatistician and a pharmacovigilance representative, will operate independently. The DSMB is empowered to recommend changes in the design of the study to ensure the safety of the patients and scientific integrity of the study.

Withdrawal

Participation is strictly voluntary and a patient or their legal representative may withdraw the patient from the study at any time without providing an explanation. This will not affect his/her right for future medical care. If a patient would withdraw from the study, the date, circumstances and any reason provided will be documented on the withdrawal page of the eCRF. No data obtained after withdrawal of consent will be recorded on eCRFs, unless the patients consents to the use thereof. For safety analysis, the patient's outcome status (dead or alive) at day 90 will be collected. For the main efficacy analysis, these patients will be excluded. In order to rule out that patient withdrawal is linked to treatment, a sensitivity analysis will be conducted assigning missing endpoint data with the worst possible value (i.e., worst possible value for patients in the treatment group, the best possible value for patients in the control group). In addition, an analysis will be conducted where missing data points will be imputed using interpolation or extrapolation, if applicable.

Study period

The study started enrolling patients in December 2017. The estimated study enrolment completion date is anticipated in the first half of 2019. Please note that this manuscript was finalized prior to the interim analysis.

Ethics and dissemination

Ethics

The study was started after approval of the study protocol and all other relevant study documents by the relevant institutional review boards / independent ethics committees. The study is performed in accordance with the Declaration of Helsinki, ICH, Code of Federal regulations and all other applicable regulations. Collection of personal data is performed according to country-specific regulations.

Confidentiality

After written informed consent has been obtained, patients will be assigned a unique 6-digit patient identification number. This allows identification of patients, while maintaining patient confidentiality. The investigators, designated CRO and sponsor and all other involved parties will preserve the confidentiality of all patients taking part in the study, in accordance with ICH-GCP and local regulations. Confidentiality of all patient identities will be maintained, except during source data verification when monitors,

auditors and other authorized agents of the sponsor or its designee, the ethics committee or any other applicable regulatory authorities are granted direct access to the study patient's original medical records. No material bearing a patient's name will be kept on file by the CRO or sponsor. The code list with treatment allocations (randomisation list) is stored separately from the sponsor at the data management vendor (CRO) during the course of the study. These data management vendors will provide all relevant data (pseudonymised) to the sponsor after the end of the study. In addition, sets of sealed envelopes with randomisation codes are kept at the site for emergency unblinding, with the DSMB and with the party responsible for reporting SUSARs as required by regulatory agencies. Data retained from this study will be protected in accordance with all applicable legal requirements. Information about study patients will be kept confidential and managed according to the requirements of EU-directives 2001/20/EC, 2005/28/EC and 2003/63/EC, and relevant national and local legislation. All ongoing subjects signed the informed consent form (including the data protection part) and additionally the 'Information letter for ongoing patients' regarding the new GDPR (DSGVO, Germany). All patients have been informed by investigators before they signed these documents.

Data access

The following parties have access to the data: sponsor, sites and selected vendors (data management, pharmacovigilance). Individual patient data may be used by site investigators for publication in agreement with the sponsor. Please note that the confidentiality section also specifies some external parties that may access data (regulatory authorities, etc.).

Sample storage

A biobank for biomarkers is implemented and samples are stored for potential future use.

Study monitoring

The study is monitored by a clinical monitor, who will visit the investigator and study sites at periodic intervals in addition to phone, letter and e-mail contact. The monitor will follow the study closely through reviewing of study records and source documents, and will determine if the reported data are accurate and complete.

Dissemination policy
The data of the study will be reported at scientific meetings and published in a peer-reviewed scientific journal, regardless of the results on outcome, in accordance with the good publication practice guideline of the international society for medical publication professionals. The sponsor and the investigator and other individuals who have expertise in the area and who are willing to interpret the data and write or review articles and presentations will form a publication steering committee to oversee the preparation of articles and presentations from this study.

Discussion
The development of new therapies for the treatment of sepsis and septic shock has proven to be a challenging task over the last decades. Many trials have investigated potential adjunctive therapies, predominantly focussing on anti-inflammatory agents. Unfortunately, this enormous effort put into dozens of clinical trials has not yielded compounds with clinically relevant beneficial effects. This can be explained by many factors, such as heterogeneous study populations and difficulties in selecting patients who may best benefit from an intervention. Also, the timing of the intervention, inappropriate outcome measures and the complexity of the disease with multiple pathways of injury hamper clinical research in sepsis patients[5,28].

Importantly, when antibodies were used, most interventions were based on complete neutralization of the target. However, physiology probably is more balanced as some targets can exert both beneficial and detrimental effects, often even simultaneously. This may also represent a major contributing factor to the failure of many therapies to improve outcome witnessed in the last decades. Along these lines, it might be argued that a partially neutralizing therapy is more effective than total neutralization. The AdrenOSS-2 trial is an innovative, biomarker driven trial with a novel, supposedly clinically relevant efficacy endpoint.

Patient heterogeneity is a substantial contributor to the difficulties in identifying effective therapies for sepsis. Patient selection is innovative in this study for two reasons. First, a more homogeneous subgroup of sepsis patients is selected, based on the combination of presence of early signs of shock, that is, requiring vasopressor support, as well as elevated concentration

of the biomarker bio-ADM. Selecting patients in the early phase of septic shock should select patients with preventable organ dysfunction compared to patients for whom septic shock and need of vasopressors lasted more than 12 hours. Furthermore, as previously described, measuring bio-ADM at baseline correlates strongly with the need for organ supporting therapy and mortality[14,15,17,18]. Therefore, including bio-ADM as an inclusion criteria likely allows for better selection of patients who need vasopressors and have a poor outcome. Combining need of vasopressor and high bio-ADM may contribute to obtaining a more homogeneous population of patients whom may benefit most from this adjunctive sepsis therapy. To our knowledge, this is one of the first precision medicine study in sepsis patients[29].

ADM is a key vasoactive peptide involved in several important pathways in sepsis, which makes it an attractive therapeutic target in sepsis[10]. It has previously been described as a double-edged sword in sepsis[30]. On vascular smooth muscle cells, ADM exerts vasodilatory effects and thereby induces vasodilation and hypotension[11-13]. This effect of interstitial ADM may exacerbate the severity of shock and may lead to organ hypoperfusion and organ dysfunction. In contrast, ADM present in the circulation exerts potent endothelial barrier stabilizing effects, reducing vascular leakage that may improve survival, as was demonstrated *in vitro*[6,7,31,32] and *in vivo* in animal models of sepsis and systemic inflammation[8,9,33,34]. However, direct administration of ADM during sepsis poses several limitations. Because of a short half-life[11], continuous infusion of ADM would be required. In addition, due to ADM's potent vasodilative effects, ADM-induced hypotension might be an issue, which might further aggravate shock in septic patients. A non-neutralizing antibody might attenuate ADM's vasodilatory effects on VSMCs and potentiate ADM's effects on endothelial cells.

Adrecizumab, a *non-neutralizing* ADM-binding antibody, is one of the first therapies specifically aimed at improving vascular endothelial barrier function, and represents a new candidate drug for the treatment of septic shock. A detailed description of Adrecizumab's supposed mode of action is described elsewhere[35]. Briefly, during sepsis, increased concentrations of ADM in the interstitial compartment are thought to contribute to hypotension. Adrecizumab, which is confined to the blood compartment, shifts the distribution of ADM away from the interstitium towards the blood, by preventing diffusion of bound ADM[35]. This results in a strong increase of (bound) ADM concentrations

in the blood[22,23,24], where it, being bound to a non-neutralizing antibody, interacts with receptors on endothelial cells and reduces vascular leakage and tissue edema. At the same time, concentrations in the interstitium are reduced through this mechanism, leading to less vasodilation and subsequent hypotension. This increase in plasma ADM concentration was observed in a rapid and dose dependent manner upon intravenous administration of Adrecizumab, both in animals and in humans[21-24]. Through reducing vascular leakage, tissue edema and hypotension, Adrecizumab could increase tissue perfusion and improve the prognosis of sepsis patients, whereas it might also reduce the use of vasopressors, thereby limiting potential adverse effects of vasopressors[36,37].
Adrecizumab, administered as a single intravenous dose (due to its long half-life of 14 days), showed promising results in preclinical studies of systemic inflammation and septic shock, including attenuation of vascular leakage, lower vasopressor infusion rates and less organ dysfunction, related to improved survival[19-21].

Substantial effort has been directed at reducing mortality in sepsis patients. Nevertheless, all major sepsis trials have failed to improve survival. Although survival is a clear and relevant end-point, it may be too insensitive to demonstrate a beneficial effect of a novel intervention. Therefore, novel endpoints beyond all-cause mortality should be considered[38]. The use of composite endpoints allows for more nuanced assessment of morbidity and mortality. A new composite endpoint, the SSI, is used in the present study as the primary efficacy endpoint. The SSI is a composite index reflecting days on organ supportive therapy (hemodynamics, pulmonary), days with organ dysfunction (renal), as well as all-cause mortality. These organ systems were improved by Adrecizumab administration in preclinical models, and support of these organ systems defines ICU care, indicating that a therapeutic effect is of clinical relevance. The SSI is thought to allow for earlier and more sensitive observations of possible clinically relevant beneficial effects of Adrecizumab compared to more traditional primary efficacy endpoints.

Potential limitations of the study include strict inclusion and exclusion criteria and a short window for patient inclusion (within 12 hours following vasopressor therapy). These limitations result in a more homogenous study population, but they may make recruitment more difficult and limit the generalisability of the results.

Conclusion

Despite the exponential increase of knowledge gathered in the last decades pertaining the pathophysiology of septic shock, this has not translated to effective therapeutic interventions and as a consequence, this condition remains to have an unacceptable high morbidity and mortality. The AdrenOSS-2 trial is one of the first personalised medicine trials in patients with septic shock, aimed at characterising the safety and efficacy of the ADM-binding antibody Adrecizumab in patients with septic shock with elevated concentrations of bio-ADM. The trial incorporates a number of innovative features such as biomarker-guided patient selection and a novel efficacy endpoint in its design to avoid ptifalls of previous sepsis trials. Adrecizumab represents a promising approach to treat this lethal syndrome. The results of this proof-of-concept and dose-finding phase II trial are eagerly awaited, and will importantly aid the design of future trials with this drug.

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Chapter 14

Summary

The primary aim of this thesis was to investigate the potential of the non-neutralizing, adrenomedullin-binding antibody Adrecizumab as a novel treatment strategy for septic shock. To do so, we first thoroughly evaluated the current knowledge on endogenous adrenomedullin, including intracellular pathways, effects of adrenomedullin on organ systems affected by sepsis, and the potential use of adrenomedullin-targeted therapies in part I. In this part, we also evaluated the existing literature on adrenomedullin in heart failure and reviewed the experimental human endotoxemia model which we used for translational research later in this thesis. In part II, we studied the kinetics of circulating endogenous adrenomedullin and its relation with outcome in both a general critically ill patient population and in sepsis patients. Next, we performed preclinical studies to assess the safety, efficacy and mode of action of Adrecizumab in part III. Finally, we focused on the clinical evaluation of Adrecizumab in part IV.

In the general introduction and outline of this thesis (**chapter 1**), we provided a brief historical perspective on sepsis and focussed on the vascular endothelial barrier as a potential target for pharmacological interventions. In addition, we introduced the vasoactive peptide adrenomedullin and the novel humanized adrenomedullin-binding antibody Adrecizumab.

Part I: Background

In **chapter 2**, we reviewed the existing literature on adrenomedullin and sepsis. Briefly, adrenomedullin is a peptide hormone that is predominantly produced by endothelial and vascular smooth muscle cells. Several factors present in patients suffering from sepsis stimulate adrenomedullin synthesis, including hypoxia, catecholamines, lipopolysaccharide and various cytokines. As such, increased concentrations of adrenomedullin are indeed observed in septic patients, which correlate with disease severity and mortality. Adrenomedullin has a relatively short half-life of 22 minutes, and is removed from the circulation through proteolytic degradation or through internalisation upon binding with its receptors. Although strong vasodilation was the first discovered biological effect of adrenomedullin, more recent studies revealed that it exerts a multitude of actions, including anti-inflammatory, anti-apoptotic and anti-microbial effects, as well as strong endothelial barrier-protection properties. In this review, we also provided an extensive overview of preclinical *in vitro* and *in vivo* studies that have investigated the potential therapeutic use of adrenomedullin in animal models of systemic inflammation and sepsis. In

many of these studies, administration of adrenomedullin exerted beneficial effects on outcome. This might be attributed to its barrier-enhancing effects upon ligation with endothelial cells, and we describe the involved pathways in greater detail. However, there are likely some drawbacks to adrenomedullin therapy that make it less attractive as a potential therapeutic agent in patients. The most important one is potent vasodilation, which causes hypotension and could further aggravate shock in patients with low blood pressure due to sepsis already. Several attempts have been made to negate the hypotensive effects of adrenomedullin and enhance its beneficial effect, for example through co-administration of adrenomedullin with adrenomedullin-binding peptide-1 (AMBP-1), administration of PEGylated adrenomedullin and by complete or partially (i.e. non-neutralizing) inhibiting antibodies (Adrecizumab).

In **chapter 3**, we focussed on the vascular effects of adrenomedullin in sepsis and of preclinical studies with Adrecizumab and its murine predecessor HAM1101. In addition, we formulated a completely novel hypothesis on the mechanism of action of Adrecizumab. The observation that plasma adrenomedullin levels are increased upon administration of the antibody is a fundamental aspect of this hypothesis. In short, we theorised that Adrecizumab induces a shift of adrenomedullin from the interstitium into the blood compartment, since the small peptide adrenomedullin – which normally is able to defuse freely over the endothelial barrier – cannot do this anymore upon binding with the much larger antibody in the circulation. This leads to increased circulating levels of adrenomedullin complexed with Adrecizumab. Since Adrecizumab only partially inhibits adrenomedullin signaling, we hypothesize that there is 'net' enhanced adrenomedullin signaling on endothelial cells, thereby exerting endothelial barrier-stabilizing effects. At the same time, the draining of the vascular interstitium (as Adrecizumab is retained in the circulation and binds ADM) may lead to lower interstitial levels of adrenomedullin following treatment with Adrecizumab. As a therapeutic property, this may attenuate vascular smooth muscle cell-mediated vasodilation and improve hemodynamic stability in patients with septic shock. In addition, we show that Adrecizumab prolongs the half-life of circulating adrenomedullin, likely by protecting the N-terminal epitope from proteolytic degradation. This aspect may also be partially responsible for the observed increased circulating levels of adrenomedullin.

In **chapter 4**, we commented on a review by others detailing vasopressor treatments. Herein, Adrecizumab is mentioned as an adrenomedullin-blocking compound, which does not accurately describe Adrecizumab's purported mechanism of action. In this letter, we briefly summarized our aforementioned hypothesis.

In **chapter 5**, we reviewed the existing literature on heart failure and adrenomedullin, and speculated on the potential of Adrecizumab as a treatment option for patients with heart failure. Interestingly, increased concentrations of adrenomedullin and related peptides (such as the inactive mid-regional pro-adrenomedullin [MR-proADM] peptide) are observed in heart failure patients and correlate with adverse outcome and fluid overload. Because higher levels of adrenomedullin appear to reflect residual tissue congestion, which in turn is related to impaired outcome and a higher likelihood for hospital readmission, we propose that adrenomedullin levels might guide diuretic treatment and/or discharge decisions. Finally, we discuss the potential of Adrecizumab as a treatment strategy for heart failure patients. Because adrenomedullin has potent endothelial barrier-stabilizing properties and also inhibits aldosterone secretion, the enhancement of circulating levels of adrenomedullin by Adrecizumab treatment might be advantageous in this group of patients.

A thorough evaluation of the literature on the experimental human endotoxemia model utilized in chapter 12 of this thesis is provided in **chapter 6**. In this model, healthy volunteers are administered purified endotoxin (lipopolysaccharide) intravenously. This induces a safe, transient, and well-controlled systemic inflammatory response, which bears several similarities to the inflammatory response observed in sepsis patients. Because this model can be used in a highly standardized and reproducible manner, the model allows researchers to investiage the underlying mechanisms of systemic inflammation and potential interventions in humans *in vivo*. We describe several strengths and limitations of this translational model, possible study designs and organ-specific changes elicited by endotoxin infusion.

Part II: Adrenomedullin as a biomarker in the critically ill

A novel assay has recently been developed to measure circulating levels of biologically active adrenomedullin (bio-ADM). This assay enables rapid and highly specific measurements of bio-ADM using a sandwich immunoassay and has several advantages over previously used radioimmunoassays. Furthermore, it yields actual bioactive ADM concentrations and not surrogates such as the inactive precursor MR-proADM. The assay has potential as a tool to monitor tissue congestion, predict progression of disease, and stratify patients to specific treatments (for example new compounds that target the adrenomedullin system, such as Adrecizumab).

In **chapter 7**, using data from the FROG-ICU study (French and European Outcome reGistry in Intensive Care Units), the relation between bio-ADM levels at admission and outcome parameters was assessed in a critically ill ICU population (including both septic and non-septic ICU patients). Over 2000 patients were enrolled. The highest concentrations of bio-ADM at admission were found in patients with septic shock (median 99.7 [IQR 62-194.4] pg/mL), whereas the lowest levels were observed in patients with neurological disorders (median 28 pg/mL [IQR 18-48]). Bio-ADM concentrations were independently associated with the need for organ support, renal replacement therapy as well as the use of inotropes and/or vasopressors, even in patients who were not on inotropic/vasopressive support at baseline. In addition, elevated bio-ADM levels were independently associated with prolonged length of stay and 28-day mortality. These observational data illustrate that circulating concentrations of endogenous ADM have prognostic value in a general ICU population, especially in those patients that suffer from systemic inflammation.

A second study was performed in patients with sepsis and septic shock (AdrenOSS study; The prospective observational multinational Adrenomedullin and Outcome in Sepsis and Septic Shock study), and the results are presented in **chapter 8**. Bio-ADM concentrations at admission were significantly higher in septic shock vs. severe sepsis patients (114 vs. 58 pg/mL). In addition, bio-ADM levels at ICU admission correlated significantly with initial organ dysfunction (SOFA), need for organ support, and 28-day mortality. Moreover, patients with bio-ADM concentrations >70 pg/mL at admission were more likely to require renal replacement therapy and had a more positive fluid balance compared to patients with bio-ADM levels

<70 pg/mL. Bio-ADM had additional value in concert with APACHE and lactate levels. Finally, in patients with bio-ADM concentrations >70 pg/mL at admission, a decrease to below 70 pg/mL on ICU day 2 was associated with a full recovery of organ dysfunction by day 7 and a 28-day mortality of 10%. In contrast, persistently elevated bio-ADM levels on day 2 were associated with prolonged organ dysfunction and a 28-day mortality of 38%. Taken together, bio-ADM has additional prognostic value compared to currently used clinical scores and lactate at ICU-admission in sepsis patients. Moreover, repeated measurements improve this prognostic accuracy even further.

Part III: Preclinical evaluation of the adrenomedullin-binding antibody Adrecizumab

Previously, the efficacy of the murine adrenomedullin-binding antibody HAM1101 (the predecessor of Adrecizumab) was demonstrated in rodent models of sepsis. We investigated the safety and efficacy of the humanized form of this antibody, known as 'Adrecizumab', in healthy animals and in preclinical models of systemic inflammation and sepsis.

In **chapter 9**, we studied the effects of Adrecizumab on vascular barrier dysfunction and survival in rodent models of systemic inflammation and sepsis. In 48 Wistar rats, pretreatment with Adrecizumab or placebo was directly followed by administration of 5 mg/kg lipopolysaccharide to induce systemic inflammation. Twenty-four hours later, rats were sacrificed and renal albumin concentrations were assessed as a measure of vascular leakage. Pretreatment with 0.1 and 2.5 mg/kg Adrecizumab significantly reduced renal, but not hepatic, albumin leakage compared to placebo. In addition, circulating levels of adrenomedullin were dose-dependently increased following Adrecizumab administration. In another experiment in 24 C57BL/6 mice, pretreatment with Adrecizumab was followed by cecal ligation and puncture (CLP) surgery to induce polymicrobial sepsis. It was demonstrated that Adrecizumab attenuated renal expression of albumin and the detrimental peptide VEGF, while concentrations of the protective protein angiopoietin-1 were augmented. Finally, survival was assessed in 60 C57BL/6 mice following CLP-induced sepsis. Pretreatment with either HAM1101 or Adrecizumab significantly increased survival compared to placebo, both when administered as single dose, as well as following repeated dosages. Note that Adrecizumab was administered before the onset of systemic inflammation and sepsis in this

chapter (pretreatment). As, in clinical practise, patients receive treatment after they become ill, this limits the generalizability.

In **chapter 10**, the effects of delayed administration of Adrecizumab on hemodynamic parameters and cardiac function were further studied in rats. Male Wistar rats underwent CLP and 24 hours later, a single dose of 2 mg/kg Adrecizumab (or placebo) was administered intravenously. Control groups included sham-operated animals and rats receiving noradrenaline with or without concurrent Adrecizumab administration. For the next 3 hours, blood pressure was measured invasively, and cardiac function was assessed hourly by echocardiography. Thereafter, animals were sacrificed and several organs were analysed for inflammatory peptides and oxidative stress. Adrecizumab significantly increased systolic blood pressure, heart rate and cardiac output compared to placebo-treated animals, to a similar extent as in rats in which CLP-induced septic shock was treated with norepinephrine only. Addition of Adrecizumab administration to norepinephrine-treated rats did not further improve hemodynamic parameters. CLP resulted in a significant increase of circulating bio-ADM levels compared to sham-operated animals (15 vs. 290 pg/mL). Administration of 2 mg/kg Adrecizumab enhanced CLP-induced bio-ADM levels greatly (1193 pg/mL). Adrecizumab treatment did not affect cytokine concentrations in various organs or myocardial Akt phosphorylation levels. However, Adrecizumab did significantly blunt CLP-induced myocardial oxidative stress.

The preclinical safety of Adrecizumab was thoroughly evaluated in rats, beagle dogs and cynomolgus monkeys, and these data are presented in **chapter 11**. Briefly, Wistar rats and cynomolgus monkeys received four repeated administration of Adrecizumab in various dosages over a period of 14 days, whereas beagle dogs received a single administration of various dosages of Adrecizumab. Safety observations included clinical signs and symptoms, mortality, extensive blood and urine analyses, as well as histopathology. The beagle study focussed primarily on hemodynamics, measured in the first 24 hours post-administration by telemetry devices. Adrecizumab was well-tolerated in tested dosages of up to 400 mg/kg/day in rats, 50 mg/kg/day in beagle dogs and 100 mg/kg/day in cynomolgus monkeys. No significant toxicologically alterations could be attributed to Adrecizumab. Toxicokinetic analysis revealed that peak concentrations were attained almost immediately after Adrecizumab administration, that peak and area-under-curve

concentrations of Adrecizumab increased in a dose-proportional manner, and that accumulation ratios of Adrecizumab were reasonably consistent. In addition, plasma levels of bio-ADM were significantly increased upon Adrecizumab administration, which did not coincide with lower blood pressure (measured non-invasively in cynomolgus monkeys and invasively in beagle-dogs).

Part IV: Clinical evaluation of the adrenomedullin-binding antibody Adrecizumab

The results from part III paved the way for clinical safety evaluation of Adrecizumab. In **chapter 12**, the safety, tolerability and pharmacokinetics/-dynamics of Adrecizumab were evaluated in healthy male volunteers in a phase I, first-in-human study and in a second study during experimental human endotoxemia. Forty-eight healthy male volunteers were enrolled in two randomized, double-blind, placebo-controlled studies. In both studies, subjects received one of three dosages of Adrecizumab (0.5, 2 and 8 mg/kg, n=6 per group) or placebo. In a second study, a bolus of 1 ng/kg of lipopolysaccharide was followed by continuous infusion of 1 ng/kg/hour (for 3 hrs) of lipopolysaccharide to induce systemic inflammation, and administration of Adrecizumab (or placebo) was started 1 hour after endotoxin bolus administration. Because the half-life of Adrecizumab was predicted to be quite long from the animal studies, subjects were followed for a 90-day period. Excellent safety profiles of Adrecizumab were demonstrated both during non-inflammatory, as well as inflammatory conditions. Pharmacokinetic analyses revealed results typical for monoclonal antibodies, including proportional increases of the maximum plasma concentrations with increasing dosages, a small volume of distribution, low clearance rate and a terminal elimination half-life of approximately 14 days. Systemic inflammation (in the volunteers of the endotoxemia trial) did not relevantly alter the pharmacokinetics. In line with the animal data presented in chapters 9-11, Adrecizumab induced a swift, profound and dose-dependent increase of circulating bio-ADM levels (see also chapters 3 and 4 for our hypothesis on this observation). Importantly, Adrecizumab did not affect the endotoxemia-induced decrease in mean arterial pressure or increase in body temperature, nor did it influence cytokine kinetics. This was not surprising, as the design of the study focussed on pharmacokinetics and safety of Adrecizumab during inflammation and therefore Adrecizumab was administered 1 hour after

initiation of LPS infusion, during which the immune response was already fully mounted. As a consequence, Adrecizumab was not expected to modulate the innate immune response. Nevertheless, endotoxemia-induced flu-like symptoms were significantly attenuated in the 8 mg/kg dosage group, while a trends towards significance were observed in the lower Adrecizumab dosage groups.

Based on the results of this preclinical and translational research in humans, in **chapter 13**, the study protocol of the AdrenOSS-2 study is presented. In this double blind, placebo-controlled, randomized, multicenter, proof of concept and dose-finding phase II first-in-patient clinical trial, the safety and efficacy of Adrecizumab will be investigated in patients with septic shock and elevated adrenomedullin concentrations. The primary endpoint is the safety and tolerability of Adrecizumab over a 90-day period. Some notable features of this study are biomarker-guided patient selection (bio-ADM >70 pg/mL at ICU admission) and a novel composite efficacy endpoint (the Sepsis Support Index). The former is aimed at selecting patients with a high likelihood of an impaired outcome who are expected to benefit most from Adrecizumab therapy. The latter combines all-cause mortality and organ dysfunction over a 14-day period, and is expected to be more sensitive for assessment of treatment efficacy than mortality only. A total of 300 patients will be enrolled at approximately 30 sites in the European Union, and two dosages of Adrecizumab will be investigated (2 and 4 mg/kg). The study started in December 2017 and results are eagerly awaited.

Chapter 15

General discussion and future perspectives

The peptide hormone adrenomedullin has been referred to as a 'double-edged sword' in sepsis[1]. Elevated levels likely exert endothelial barrier-stabilising effects, but simultaneously may contribute to more pronounced vasodilation and hypotension. In this thesis, we show that circulating levels of biologically active adrenomedullin (bio-ADM) reflect disease severity, need for organ support, and mortality in a general ICU population and in sepsis patients, and therefore has potential use as a prognostic biomarker as well as for patient stratification. In addition, we demonstrated promising effects of adrenomedullin-binding by the novel antibody Adrecizumab in animals and humans, and proposed a mechanism of action. In this final chapter, we discuss the findings described in this thesis and present future perspectives.

Potential use of bioactive adrenomedullin as a biomarker in critically ill patients

The term biomarker (biological marker) is defined as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention'[2]. In clinical practice, biomarkers are already used in the form of (routine) laboratory tests, and can aid in screening (detecting subclinical disease), diagnosis (confirming overt disease), prognosis or staging (predicting the disease course/assessing severity), patient stratification (selecting patients which may benefit most from a given intervention), and titration of therapies (with regard to efficacy and/or toxicological side-effects). In the field of sepsis, which is characterized by a very heterogeneous patient population and a highly complex pathophysiology, it has been proposed that a more personalized approach would be required to move the field forward, with medical decisions and treatments being tailored to the individual patient[3]. Biomarkers could potentially play an important role in this process, which is also known as 'precision medicine'.

In line with previous work, we showed that bio-ADM has potential prognostic value in several critically ill patient categories, as it independently reflected disease severity, positive fluid balance (possibly reflecting capillary leakage that arises through endothelial barrier disruption), need for organ support and mortality. Notably, in patients who did not yet receive vasopressors during ICU admission, bio-ADM predicted later vasopressor requirement. Nevertheless, it is important to critically assess bio-ADM's advantages and limitations, and to compare bio-ADM to other biomarkers related to the adrenomedullin

pathway, as well as to currently used means of prognostication (e.g. clinical criteria and blood lactate levels[4]). In addition, possible implications for clinical practice and stratification for clinical trials need to be discussed.

Let us first consider the technical robustness of the bio-ADM assay. This was thoroughly evaluated by others recently[5], showing a high specificity of the assay for biologically active adrenomedullin. No cross-reactivity with the putative adrenomedullin-binding complement factor H, inactive glycine-extended adrenomedullin or related peptides such as adrenomedullin-2, calcitonin gene-related peptide, calcitonin or amylin was observed. In addition, the assay showed stable results even when plasma samples were stored for up to 24 hours at ambient temperature and over multiple freeze-thaw cycles. Finally, the lower and upper levels of detection and quantification allows researchers to assess bio-ADM levels over a wide range of diseases with varying plasma levels.

Midregional pro-adrenomedullin (MR-proADM) represents the most prominent alternative biomarker of the adrenomedullin system, and has often been used as a surrogate marker for adrenomedullin, partially due to technical difficulties related to the measurement of the latter in the past (e.g. need for large sample volumes, difficult preanalytical sample extraction and long incubation times). At first glance, this makes sense, as both MR-proADM and bio-ADM are derived from the same precursor peptide in a similar ratio (**Figure 1**). Also, several studies have demonstrated that MR-proADM levels are elevated in critically ill patients and correlate strongly with mortality, indicating that it also has potential as a biomarker, for example in triage in the emergency department[6,7]. However, MR-proADM measurements do not take into account that a significant amount of adrenomedullin is circulating in its 'inactive' glycine-extended form[8]. In addition, clearance kinetics of MR-proADM and bio-ADM likely differ (MR-proADM has a longer half-life). Therefore, bio-ADM measurements may better reflect the activity of the ADM system and may correlate better with the clinical status of the patient. So far, only one study assessed bio-ADM and MR-proADM concurrently, and showed that the correlation of bio-ADM with 28-day mortality in sepsis patients was higher compared to MR-proADM[9]. However, the fact that MR-proADM levels were only measured in a subset of patients represents an important limitation of this study. Given the lack of other studies that measured these peptides simultaneously, no definite conclusions can be

drawn. Future research is warranted to compare bio-ADM and MR-proADM head-to-head in order to determine whether the theoretical benefits of bio-ADM indeed translate into a better reflection of the clinical status of patients and exerts superior prognostication.

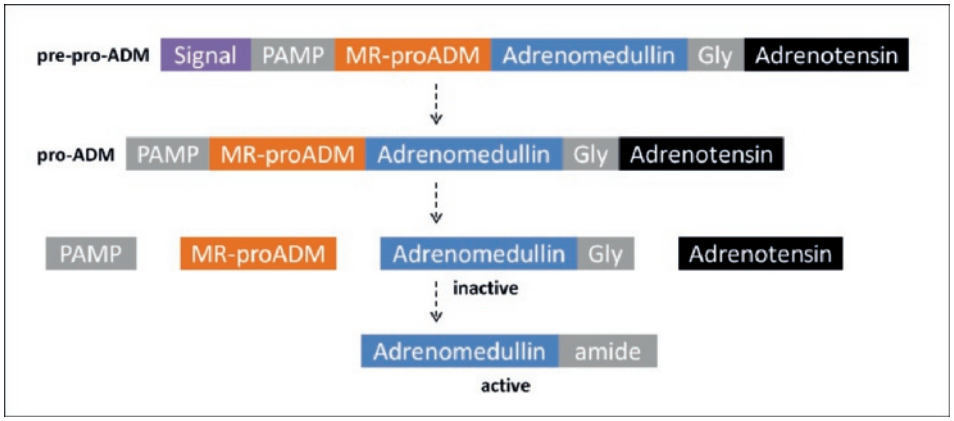

Figure 1. Schematic representation of adrenomedullin's biogenesis.

Another important aspect is to determine in which patient groups bio-ADM has prognostic value: are (changes in) bio-ADM levels relevant for all critically ill patients, or are there exceptions? In the study presented in chapter 7 of this thesis, which was performed in a general ICU population, bio-ADM correlated strongly with disease severity, organ support and outcome. Although this was already established for sepsis patients[9,10], we were able to extend this observation to a general ICU population including subgroups of acute respiratory failure, cardiac arrest and cardiogenic shock. In our study, across all patient categories, bio-ADM levels were highest in septic shock. This is most likely related to the severity of the systemic inflammatory response, as a previous study demonstrated a correlation between pro-inflammatory plasma cytokine levels and adrenomedullin concentrations in patients with both infectious and non-infectious systemic inflammation[11]. In accordance, in our study, patients with a higher bio-ADM level showed more systemic inflammation (e.g. higher CRP concentration and leukocyte counts), while bio-ADM levels were the lowest in patients with neurological disorders and did not correlate well with outcome in this specific subgroup. These observations support the notion that systemic inflammation and bio-ADM are related to endothelial dysfunction. The interplay between bio-ADM release from the

endothelium and endothelial dysfunction in the relation to the prognosis of patients warrants further exploration, as the endothelium is both an important source, as well as target of adrenomedullin[12,13]. In addition, future studies that measure adrenomedullin and interventions that target this pathway should focus on patients with profound systemic inflammation and/or heart failure.

Use of bio-ADM as a biomarker is only useful if it has additional value compared to existing clinical scores or for example, lactate levels. In the AdrenOSS study presented in chapter 8, the prognostic value of bio-ADM at admission for the primary endpoint 28-day mortality was only moderate, with a C-index (comparable to the AUROC) of approximately 0.7, similar to that of lactate, SOFA and APACHE II. However, for initial mortality prediction, many risk scores or individual biomarkers show AUCs of around 0.7, possibly due to the fact that many critically ill patients eventually die of causes not directly related to the initial disease. Note that bio-ADM's prognostic value for mortality was independent from the aforementioned variables and other covariates, and that it showed added prognostic value when combined with these variables (which was also observed in chapter 7 of this thesis). This illustrates that a combination of biomarkers improves prognostication at ICU admission. A combination of several biomarkers and/or clinical scores is likely superior, which represents an opportunity for further research with bio-ADM. Interestingly, repeated measurement of bio-ADM later in the disease course (48 hours after the first measurement) showed interesting results (described in chapter 8). Survival of patients with high initial bio-ADM levels which subsequently recovered towards normal concentrations was comparable to that of patients already displaying low bio-ADM levels at admission. In contrast, patients with a persistently high bio-ADM level had poor survival. Apparently, there is a group of patients that has high initial bio-ADM at admission, but nevertheless makes a quick recovery. Therefore, early bio-ADM measurements may not always yield reliable prognostic information and a repeated measurement may be of more value. The reasons behind these observations remain to be explored; perhaps the source of sepsis (e.g. pneumonia or abdominal infection), other timely interventions (e.g. antibiotic treatment, fluid resuscitation), or other factors are involved. Finally, it is of no use to compare bio-ADM with other commonly used biomarkers such as procalcitonin, C-reactive peptide and/or leukocytes, because these are used as diagnostic biomarkers for infection vs. sterile inflammation, and do not have prognostic value. Bio-ADM has not yet been compared head-to-

head to other experimental biomarkers for sepsis prognostication, of which there are many.

Another aspect that warrants discussion is the 70 pg/mL cut-off value for bio-ADM, which was first proposed in a 2014 paper[9]. The scientific rationale for choosing this concentration was described somewhat ambiguous (the paper only stated that it was close to the 99th percentile of the normal range [43 pg/mL]), but nevertheless, it proved to correlate well with organ dysfunction/support and mortality. After the initial study, three subsequently performed studies, including ours (chapters 7 and 8 of this thesis)[10], used the 70 pg/mL cut-off value and confirmed that it indeed exerts prognostic value. However, it may be the case that a different cut-off value is more optimal for risk stratification, depending on the specific population, setting and diseases studied. In the AdrenOSS study described in chapter 8, additional ROC analyses were performed to assess optimal cut-off values for 28-day mortality. The Youden cut-off value of bio-ADM at admission was 102 pg/mL for severe sepsis and septic shock patients combined (sensitivity 68%, specificity 67%), 102 pg/mL for severe sepsis patients (sensitivity 58%, specificity 79%), and 99 pg/mL in septic shock patients (sensitivity 71%, specificity 52%), compared to a sensitivity of 77% and specificity of 49% for the predefined cut-off value of 70 pg/mL in severe sepsis and septic shock patients combined. In the Frog-ICU study, a cut-off value of 75 pg/mL was found using sensitivity analysis. For AdrenOSS-2, a cut-off value of 70 pg/mL was adopted to aid in selecting the most severely ill patients with a higher likelihood to develop organ dysfunction, vasopressor requirement, vascular leakage and a higher mortality: patients who may benefit most from treatment with Adrecizumab. Future studies should further evaluate different bio-ADM cut-off values for different patient populations and diseases.

Altogether, bio-ADM is a promising biomarker with prognostic value in critically ill patients with various diseases, such as sepsis[9,10,14], cardiogenic shock[15], acute myocardial failure[16] and acute heart-failure[17,18]. It is of interest to compare groups of patients based on bio-ADM levels, however, it remains to be determined to what extent the prognostic value of bio-ADM will actually influence bedside management of sepsis patients. Comparable to the use of APACHE score and lactate, in these patients that are already in an environment with a continuously high level of monitoring where discharge decisions primarily depend on whether a patient is weaned off organ support,

individual policy decisions should not be dependent on a single biomarker. Ultimately, the real value of (serial measurements of) bio-ADM may lie in initiation and/or guidance of specific therapies, such as diuretics for heart failure (speculated on in chapter 5 of this thesis), identifying patients with endothelial barrier disruption who may benefit from future endothelial barrier-stabilising drugs, or selecting patients for studies or stratification of patients within a study with novel therapies that target the adrenomedullin system (such as the AdrenOSS-2 study presented in chapter 13).

Adrenomedullin as a double-edged sword in sepsis

We want to address the fact that several authors have referred to adrenomedullin as a 'double-edged sword' in sepsis. It is interesting to speculate whether endothelial barrier-stabilising and vasodilatory effects of adrenomedullin are exerted at bio-ADM concentrations commonly observed in septic patients (median of 100-125 pg/mL, see chapters 7 and 8 of this thesis)[9,10]. In order to shed more light on this issue, we reviewed the literature pertaining concentration-dependent effects of ADM observed in *in vitro* and *in vivo* studies.

Starting with endothelial barrier-stabilising effects, in *in vitro* studies with (human) endothelial cell monolayers, stabilising effects were observed using adrenomedullin concentrations of 0.01-0.1 μmol/L[19,20], which roughly translates to 60000-600000 pg/mL, much higher than those observed in septic shock patients. Unfortunately, comparing effective concentrations between *in vitro* and *in vivo* studies is notoriously troublesome, and therefore no definite conclusions can be drawn based upon this. *In vivo* studies showed that treatment with 24-500 μg/kg/hr adrenomedullin improved survival and reduced endothelial hyperpermeability in various animal models[21-23]. However, plasma adrenomedullin concentrations were unfortunately not measured, and in our opinion it is too speculative to convert these dosages to plasma concentrations. Note that we did observe attenuated vascular leakage (as a measure of endothelial barrier stabilisation) at ADM concentrations of approximately 100 pg/mL and higher in chapter 9 of this thesis.

Next, let us consider the cardiovascular effects of adrenomedullin. In contrast to endothelial effects, in vitro work showed that vasodilation of rat carotid arteries was attained by much lower adrenomedullin concentrations, starting at approx. 60 pg/mL[24]. In vivo in humans, cardiovascular effects were demonstrated at dosages of approximately 150 pg/min/mL[25], and in other

studies, at plasma concentrations of roughly 180 pg/mL and 70 pg/mL[26,27]. These data indicate that adrenomedullin likely exerts vasodilatory effects leading to hypotension at concentrations that are commonly observed in septic shock patients.

Taken together, these (rough) calculations suggest that adrenomedullin – in concentrations commonly observed in septic patients – very likely exerts cardiovascular effects, including hypotension, but may not accomplish endothelial barrier stabilisation. This is in line with the fact that in septic patients, higher adrenomedullin concentrations are associated with worse outcome, hypotension and vasopressor requirement. The term "double-edged sword" may therefore not be appropriate in sepsis patients. Furthermore, this emphasizes the need for therapies that enhance adrenomedullin's beneficial effects and negate its detrimental ones. Adrecizumab appears to represent a prime example of such a therapy.

Adrecizumab as a novel therapy for sepsis

After promising studies with the murine predecessor of Adrecizumab (HAM1101)[28,29], a humanized form of this antibody was developed and named Adrecizumab. We evaluated the efficacy and safety of Adrecizumab in preclinical animal studies and two clinical studies in healthy volunteers (chapters 9-12). Furthermore, we formulated a hypothesis on Adrecizumab's mechanism of action (discussed in chapters 2-4), and we published the study protocol for a phase II study with Adrecizumab in patients with septic shock (chapter 13). These studies provide the basis for further discussion and potential future studies.

Let us first consider the proposed mechanism of action of Adrecizumab. Central to this hypothesis is the observation that adrenomedullin-binding with Adrecizumab causes a shift from the vascular interstitium to the blood compartment resulting in a profound increase of circulating concentrations of its target peptide adrenomedullin. We showed that this increase is not due to enhanced synthesis (as MR-proADM concentrations remained stable), but instead hypothesised that this is 1) due to a redistribution from the tissue/interstitium to the blood and 2) enhanced half-life due to protection from proteolytic degradation that normally occurs via the N-terminal epitope of adrenomedullin (where Adrecizumab also binds). Interestingly, Adrecizumab is not the first antibody described in literature

to enhance circulating levels of its target peptide. It is well known that antibody binding can lead to redistribution of a ligand from the tissue to the blood, with significant decreases in tissue ligand concentrations[30]. Prime examples of this are antibody fragments used to treat patients with digoxin-intoxication. Shortly after administration of anti-digoxin antibody fragments, plasma concentrations of digoxin increase strongly due to redistribution of digoxin to the blood, and since the majority of digoxin is bound to antibody fragments, it can no longer interact with its biological tissue receptor and thus reverses cardiac digoxin toxicity[31]. Likewise, we propose that Adrecizumab lowers tissue/vascular interstitial levels of adrenomedullin, which in turn attenuates adrenomedullin's vasodilatory effects on vascular smooth muscle cells located at the abluminal side of blood vessels. This observation is supported by a study presented in chapter 10 of this thesis, in which blood pressure improved after Adrecizumab administration in a model of murine sepsis, possibly through the aforementioned mechanism. Importantly, in contrast to most antibodies, Adrecizumab does not completely inhibit interaction of adrenomedullin with its biological receptor, and thus can still induce signalling in endothelial cells located on the luminal side of the blood vessel[28,32]. Therefore, we hypothesise that a strong increase of plasma levels results in 'net' increased signaling in endothelial cells, resulting in activation of intracellular pathways that enhance endothelial barrier structure (reviewed in chapter 2), which may explain beneficial effects related to capillary leakage that we observed (chapter 9)[33]. For future research, it would be interesting to 1) show that adrenomedullin levels in the interstitium indeed decrease upon administration of Adrecizumab, for example by measuring interstitial adrenomedullin concentrations in vivo through microdialysis, or via post-mortem techniques such as absolute quantification in tissue homogenates or immunohistochemistry to confirm our hypothesis, and 2) investigate relevant intracellular pathways in endothelial cells (we hypothesise that these are activated), as well as in vascular smooth muscle cells (we hypothesise that these are suppressed) following Adrecizumab administration.

With regard to Adrecizumab/adrenomedullin pharmacokinetics/-dynamics, there are some more interesting aspects to discuss. Adrenomedullin has been described to be cleared from the circulation via proteolytic degradation by proteases[34-36] and internalisation and degradation upon binding with its receptor, a process that also results in internalisation of the receptor[37,38]. It remains to be fully elucidated how these clearance mechanisms are affected

when Adrecizumab complexes with adrenomedullin. For example, it appears plausible that the ADM-antibody-complex becomes too large for internalisation, and therefore, the antibody-ligand complex could remain bound to ADM receptor for a long time, leading to prolonged endothelial stimulation. However, such prolonged stimulation could eventually also result in suppressed adrenomedullin signaling, either through reduced adenylyl cyclase coupling to the adrenomedullin receptor[39], or possibly alteration of adrenomedullin receptor expression, although no studies have been performed on this subject. When discussing receptor pharmacology, it also needs to be acknowledged that adrenomedullin is able to bind not to 1, but to 3 different receptors (CGRP, ADM1 and ADM2 receptors), all of which are expressed on vascular endothelial and smooth muscle cells. So far, effects of Adrecizumab were only investigated in a functional assay for the AMD2 receptor, focussing on the cAMP response, which showed partial inhibition of signaling[28]. It remains unknown what effects are for the other receptors or other second messenger systems, and whether Adrecizumab binding results in antagonistic or agonistic effects for these other receptors and pathways. In addition, next to adrenomedullin, these receptors may bind to other ligands as well, with varying affinities (a phenomenon known as 'functional selectivity' or 'biased signaling')[40]. If the affinity of these receptors for adrenomedullin is altered by Adrecizumab binding, it would be interesting to compare the receptor affinity for Adrenomedullin-adrecizumab complexes with that for other ligands, of which the effects could therefore either be enhanced or attenuated.

Of note, some of the Adrecizumab-related data presented in this thesis may appear to be conflicting with our proposed mechanism of action. In the study presented in chapter 9, in which 0.1, 0.5 and 2.5 mg/kg of Adrecizumab attenuated renal vascular leakage in a rat model of systemic inflammation, there appears to be inconsistency regarding the Adrecizumab-induced increase of adrenomedullin[33]. Animals treated with 0.5 and 2.5 mg/kg Adrecizumab showed a strong increase of adrenomedullin levels (to approximately 250 and 500 pg/mL) compared to LPS-control animals (100 pg/mL), and according to our hypothesis, increased 'net' adrenomedullin signaling on endothelial cells may be responsible for barrier stabilisation. However, the 0.1 mg/kg group also attenuated vascular leakage, while adrenomedullin levels were not additionally increased compared to LPS-control animals (peak concentrations were also approximately 100 pg/mL). Because the first measurement was performed 3 hours after Adrecizumab administration, it is possible that the

'peak' adrenomedullin concentration for this group was attained earlier and therefore missed. On the other hand, it may also indicate that the actual mechanism of action is more complex than we currently think, and that more factors play a role than adrenomedullin levels only, such as the aforementioned changes in receptor expression, receptor pharmacology/affinity, or possibly involvement of other, yet undetermined pathways. For example, adrenomedullin is known to exert immunomodulatory effects (also reviewed in chapter 2)[41]. The Adrecizumab-induced increase of adrenomedullin could therefore theoretically also exert immunomodulatory effects. A previous study in murine sepsis using the murine predecessor HAM1101 showed attenuated levels of cyto- and chemokines TNF-α, IL-6, IL-10, KC and MCP-1 at the end of the experiment[29]. However, it is unclear whether these effects were attained by direct immunomodulatory effects, or secondary to an overall improved condition of the animals, for instance due to improved hemodynamic stability or vascular barrier function. In addition, we did not find effects on plasma cytokine clearance in our experimental human endotoxemia study described in chapter 12 of this thesis, although the study was not designed to evaluate this endpoint (Adrecizumab infusion was started 1 hour after initiation of LPS infusion, when the immune response was already initiated and concentrations of various cytokines were increased). To give another example, in chapter 10 we demonstrated that delayed Adrecizumab administration in a murine sepsis model (24 hours after cecal ligation and puncture [CLP] surgery) resulted in attenuated free radical production in the myocardium, which coincided with increased cardiac output. Notably, no effects on organ cytokine levels were observed in this study, although these were only assessed at a single, relatively early timepoint (3 hours following Adrecizumab infusion). Further research should investigate if, and to what extent, adrenomedullin-binding by Adrecizumab, and the subsequent increase of circulating adrenomedullin, influences immunological and other pathways.

Overall, the pathophysiological role, especially focused on vascular and endothelial dysfunction, of the ADM-pathway in sepsis patients is convincing and Adrecizumab appears to be a promising novel drug for patients with septic shock. We demonstrated a favourable safety profile in preclinical and clinical studies. It has a plausible mechanism of action, and its effects likely include stabilisation of the endothelial barrier and attenuation of vasodilation and hypotension. Future research should focus on further exploring the mechanism of action and involvement of other pathways. In addition, it might also be of benefit in other diseases that are characterized by cardiovascular abnormalities and/or endothelial barrier dysfunction, such as heart failure, Dengue fever and other capillary leak syndromes. Most importantly, the safety and efficacy of Adrecizumab in patients with septic shock with elevated plasma levels of adrenomedullin are currently under investigation in the AdrenOSS-2 study, of which the results are eagerly awaited.

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Chapter 16

Nederlandse samenvatting

Sepsis ('bloedvergiftiging') is een veelvoorkomend probleem bij patiënten op de intensive care. Het is een ernstig ziektebeeld dat wordt veroorzaakt door een ontregelde afweerreactie van het lichaam op een infectie, zoals bijvoorbeeld een longontsteking of een urineweginfectie. In de ergste gevallen leidt dit tot een daling van de bloeddruk en orgaanschade, dan spreekt men van 'septische shock'. Ondanks moderne behandelingen met antibiotica en orgaanondersteunende therapieën (zoals bloeddrukverhogende medicatie, beademing en dialyse) overlijdt ongeveer een op de drie patiënten die lijden aan septische shock. De patiënten die het overleven hebben vaak blijvende restschade. Gezien de hoge ziektelast en sterfte, is er een dringende behoefte aan nieuwe behandelingen voor sepsis. De laatste decennia zijn tientallen experimentele behandelingen onderzocht, vaak gericht op het verminderen van de ontstekingsreactie door remming van specifieke eiwitten of biologisch processen. Helaas bleken geen van deze behandelingen effectief te zijn. Dit heeft mede te maken met de hoge complexiteit van sepsis, met activatie van meerdere biologische processen en grote onderlinge verschillen tussen patiënten.

Een belangrijk probleem bij sepsis is het disfunctioneren van bloedvaten. Allereerst treedt er vaatverwijding op, wat leidt tot de reeds benoemde lage bloeddruk. Een lage bloeddruk is gevaarlijk omdat door de slechtere doorbloeding orgaanschade (o.a. in hart, nieren, darmen, hersenen) kan ontstaan. Daarnaast is er disfunctie van de endotheelcellen die de binnenkant van de bloedvaten bekleden. Deze endotheelcellen vormen de barrière tussen het bloed en de omliggende weefsels. Deze essentiële barrière reguleert de uitwisseling van moleculen tussen deze twee compartimenten. In het geval van sepsis kan deze cellaag meer doorgankelijk worden voor grotere moleculen. Door de toename in permeabiliteit verlaat eiwitrijk vocht het bloed en ontstaat oedeem. Dit draagt eveneens bij aan een lagere bloeddruk en een verminderde zuurstofuitwisseling in de weefsels, wat verdere orgaanschade in de hand werkt.

Adrenomedulline is een eiwit dat met name geproduceerd wordt bij patiënten met septische shock. De hoogte van de concentratie is geassocieerd met een slechtere prognose voor de patiënt. Adrecizumab is een nieuw geneesmiddel dat ingrijpt op de werking van het lichaamseigen eiwithormoon adrenomedulline. Adrecizumab is een antilichaam dat mogelijk de functionaliteit (zowel de permeabiliteit als de overmatige vaatverwijding) van de bloedvaten kan

herstellen bij patiënten met septische shock. Twee experimentele studies met een voorloper van Adrecizumab lieten gunstige effecten zien op de bloeddruk en een vermindering van orgaanfalen en sterfte in proefdiermodellen waarin septische shock nagebootst werd. Deze veelbelovende resultaten hebben geleid tot de ontwikkeling van een geoptimaliseerde variant van het medicijn voor gebruik in mensen (Adrecizumab). Dit proefschrift heeft als doel om de veiligheid en effectiviteit van Adrecizumab als potentiele nieuwe behandeling voor sepsis nader te onderzoeken.

In de algemene introductie en achtergrond van dit proefschrift (**hoofdstuk 1**) wordt een historische beschrijving van sepsis gegeven. Verder worden het eiwithormoon adrenomedulline en het adrenomedulline-antilichaam Adrecizumab geïntroduceerd.

Deel I – Achtergrond

In **hoofdstuk 2** wordt een gedetailleerd overzicht gegeven van de wetenschappelijke literatuur omtrent adrenomedulline en meer specifiek, relevante effecten in de context van sepsis. Adrenomedulline is een eiwithormoon dat onder andere geproduceerd wordt door de endotheel- en spiercellen in de vaatwand. De productie van adrenomedulline wordt gestimuleerd door verschillende factoren, onder andere door zuurstoftekort en diverse ontstekingsmediatoren. Het is dan ook niet verwonderlijk dat er verhoogde concentraties adrenomedulline gemeten worden in het bloed van zieke patiënten met sepsis of septische shock. Tevens bestaat er een correlatie tussen de concentratie adrenomedulline in het bloed en de ziekte-ernst van patiënten: hoe hoger het adrenomedulline gehalte, des te zieker is de patiënt en des te groter ook de kans op overlijden. Adrenomedulline oefent diverse biologische effecten uit, onder andere vaatverwijdende, ontstekingsremmende, anti-apoptotische en antimicrobiële effecten. Daarnaast is gebleken dat het sterk beschermende effecten heeft op de endotheelbarrière en zo lekkage van vocht van de bloedvaten naar de omliggende weefsels kan voorkomen.

Verder geeft **hoofdstuk 2** een overzicht weer van studies waarbij adrenomedulline toegediend werd in dierexperimentele modellen met systemische ontstekingsreacties of septische shock. Uit deze studies bleek dat toediening van adrenomedulline gunstige effecten uitoefent op het ziekteproces, hetgeen mogelijk verklaard kan worden door de gunstige effecten

op endotheelcellen. Er kleven echter ook een aantal mogelijke nadelen aan adrenomedulline-toediening, waardoor het mogelijk minder geschikt is voor toediening bij de mens. Het belangrijkste nadeel is vaatverwijding, hetgeen kan leiden tot een lagere bloeddruk wat mogelijk ongunstig is voor patiënten die reeds een lage bloeddruk hebben (zoals patiënten met septische shock). Daarom heeft men op verschillende manieren gepoogd om adrenomedulline op een dusdanige manier te beïnvloeden, dat de bloeddrukverlagende effecten verminderd worden, terwijl gunstige effecten op bijvoorbeeld endotheelcellen versterkt worden. Voorbeelden hiervan zijn gelijktijdige toediening van adrenomedulline met adrenomedullin-binding protein-1 (AMBP-1), structurele modificatie van adrenomedulline door toevoeging van polyethyleenglycol, of toediening van adrenomedulline-bindende antilichamen (zoals Adrecizumab).

In **hoofdstuk 3** worden allereerst de vasculaire effecten van adrenomedulline tijdens sepsis beschreven en wordt gefocust op dierexperimenteel onderzoek met het adrenomedulline-antilichaam Adrecizumab. Daarnaast wordt een nieuwe hypothese geformuleerd rondom het werkingsmechanisme van Adrecizumab. Centraal in deze hypothese is de observatie dat adrenomedulline concentraties in het bloed stijgen na toediening van Adrecizumab. Er wordt gehypothetiseerd dat Adrecizumab zorgt voor een herverdeling van het beschikbare adrenomedulline, van buiten de bloedvaten (het interstitium), naar het bloed. Normaliter kan adrenomedulline, als relatief klein molecuul, zich vrijelijk verplaatsten over de vaatwand tussen het interstitium en het bloed. Echter, wanneer adrenomedulline in het bloed bindt aan het antilichaam Adrecizumab, een veel groter molecuul wat niet in staat is om zich te verplaatsen over de vaatwand, is adrenomedulline eveneens niet langer in staat om de vaatwand te passeren. Dit zorgt ervoor dat adrenomedullin als het ware 'gevangen' raakt in het bloed, wat resulteert in een stijging van adrenomedullineconcentraties in het bloed. Omdat Adrecizumab een niet-blokkerend antilichaam is (ondanks binding aan adrenomedulline is het adrenomedulline-Adrecizumab complex nog steeds in staat om een interactie aan te gaan met receptoren op endotheelcellen), zou dit gunstige effecten op endotheelcellen kunnen bewerkstelligen, hetgeen leidt tot minder capillaire lekkage en oedeemvorming. Tegelijkertijd zal de concentratie adrenomedulline in de weefsels rondom de bloedvaten afnemen, wat leidt tot minder vaatverwijdende effecten op de meer naar de buitenzijde gelegen spierlaag van de bloedvaten. Daarnaast worden er resultaten getoond waaruit

blijkt dat binding van Adrecizumab aan adrenomedulline leidt tot een verlengde halfwaardetijd van adrenomedulline, doordat het door binding aan het antilichaam beschermd wordt tegen afbraak door zogenaamde proteases, wat mogelijk ook kan bijdragen aan de geobserveerde stijging van adrenomedullineconcentraties in het bloed.

In de ingezonden brief in **hoofdstuk 4** wordt commentaar gegeven op een recentelijk gepubliceerd overzichtsartikel over bloeddrukverhogende geneesmiddelen. De auteurs van het overzichtsartikel beschrijven Adrecizumab als een middel wat de werking van adrenomedulline blokkeert, wat echter niet accuraat is. Zoals benoemd in het voorgaande hoofdstuk blokkeert Adrecizumab de werking van adrenomedulline niet. In deze brief wordt tevens de eerder vermelde hypothese over de werking van Adrecizumab samengevat.

In **hoofdstuk 5** wordt een uitstapje gemaakt naar de rol van adrenomedulline bij hartfalen. Er wordt een overzicht gegeven van de bestaande literatuur omtrent adrenomedulline en hartfalen en wordt gespeculeerd over de potentie van Adrecizumab als nieuwe behandeling voor dit ziektebeeld. Interessant is dat bij patiënten met hartfalen (net zoals bij patiënten met sepsis) verhoogde concentraties van adrenomedulline in het bloed geobserveerd worden, hetgeen correleert met een slechtere ziekte-uitkomst. Daarnaast reflecteren hogere concentraties van adrenomedulline een teveel aan extravasculair vocht (oedeem), wat ongunstig is voor patiënten met hartfalen. Daarom zou het meten van adrenomedulline in het bloed mogelijk kunnen helpen om behandeling met diuretica te sturen en een bijdrage kunnen leveren aan de beslissing om patiënten met hartfalen wel of niet te ontslaan uit het ziekenhuis. Tot slot wordt Adrecizumab als potentiele nieuwe behandeling voor patiënten met hartfalen beschreven. Omdat Adrecizumab sterke stabiliserende effecten heeft op de endotheelbarrière en oedeem kan verminderen, zou behandeling met Adrecizumab gunstig kunnen zijn voor deze groep patiënten.

Tot slot wordt in **hoofdstuk 6** een uitgebreide beschrijving gegeven van het experimentele endotoxinemiemodel bij de mens, een model dat tevens gebruikt wordt in hoofdstuk 12. In dit onderzoekmodel wordt endotoxine (lipopolysaccharide; LPS) aan gezonde vrijwilligers toegediend. LPS is een onderdeel van de celwand van E. Coli bacteriën, en toediening ervan leidt tot een kortdurende, goed-gecontroleerde ontstekingsreactie die op meerdere vlakken vergelijkbaar is met de ontstekingsreactie die plaatsvindt bij patiënten

met sepsis. Omdat dit model consistent en reproduceerbaar is, kan het door onderzoekers gebruikt worden om de afweerreactie en eventuele interventies die ingrijpen op deze afweerreactie te bestuderen bij de mens. Verder worden in dit hoofdstuk de effecten van deze ontstekingsreactie op diverse cellen en orgaansystemen beschreven, samen met de voor- en nadelen van het model. Tot slot wordt ingegaan op de verschillende manieren waarop dit model gebruikt kan worden.

Deel II – Adrenomedulline als biomarker bij kritisch zieke patiënten
Recent heeft men een nieuwe, snelle en specifieke methode ontwikkeld om biologisch-actief adrenomedulline (bio-ADM) te meten in het bloed. Deze methode heeft diverse voordelen boven het gebruik van andere methoden (zoals radioimmunoassays of het meten van het inactieve precursorhormoon MR-proADM) en geeft theoretisch een beter beeld van activatie van het adrenomedullinesysteem. Dit laatste is echter nog niet goed onderzocht. Deze nieuwe testmethode kan potentieel gebruikt worden als biomarker voor de hoeveelheid extravasculair vocht (oedeem), het voorspellen van klinische achteruitgang bij patiënten en het selecteren van patiënten voor nieuwe behandelingen (bijvoorbeeld geneesmiddelen die ingrijpen op het adrenomedulline systeem zoals Adrecizumab). In dit deel van het proefschrift worden twee studies beschreven waarin de prognostische waarde van de bio-ADM concentratie in het bloed van patiënten onderzocht is.

In **hoofdstuk 7** werd het bio-ADM gehalte in het bloed bepaald bij patiënten ten tijde van intensive care opname en is de relatie met de ziekte-uitkomst onderzocht. Dit vond plaats bij intensive care patiënten met diverse ziektebeelden, dus niet enkel in patiënten met sepsis. Hierbij werd gebruik gemaakt van data uit de FROG-ICU studie (French and European Outcome reGistry in Intensive Care Units). Meer dan 2000 IC-patiënten namen deel aan deze studie. Van alle ziektebeelden waarmee de patiënten opgenomen werden, werden de hoogste concentraties bio-ADM gevonden in het bloed van patiënten met septische shock, cardiogene shock en acuut respiratoir falen, terwijl de laagste concentraties gevonden werden in patiënten met neurologische ziektebeelden. Dit reflecteert mogelijk een meer uitgesproken ontstekingsreactie en een hogere mate van betrokkenheid van endotheelcellen bij deze ziektebeelden. Verder waren bio-ADM concentraties onafhankelijk geassocieerd met de noodzaak voor orgaan-ondersteunende behandelingen,

dialyse en het gebruik van bloeddrukverhogende medicatie. Ten slotte waren verhoogde bio-ADM concentraties geassocieerd met een langere ziekenhuisopname en een verhoogde kans op overlijden.

Een tweede biomarkerstudie (beschreven in **hoofdstuk 8**) werd uitgevoerd specifiek bij IC-patiënten met sepsis en septische shock, de AdrenOSS studie (Adrenomedullin and Outcome in Sepsis and Septic Shock). Deze studie toonde aan dat bio-ADM concentraties bij IC opname hoger waren bij patiënten met septische shock dan bij patiënten met sepsis. Daarnaast was er een relatie tussen bio-ADM concentraties bij intensive care opname enerzijds en orgaandisfunctie en kans op overlijden anderzijds. Patiënten met een bio-ADM concentratie hoger dan 70 pg/mL bij opname hadden een grotere kans dat ze dialyse nodig zouden hebben en hadden tevens een groter overschot aan extravasculair vocht (oedeem) dan patiënten met een bio-ADM concentratie lager dan 70 pg/mL. Dit had een toegevoegde voorspellende waarde bovenop andere parameters zoals de APACHE score en lactaat. Tot slot werden er op dag 2 van de intensive care opname opnieuw bio-ADM spiegels gemeten. Wanneer de bio-ADM concentratie initieel hoger dan 70 pg/mL was maar daalde tot onder 70 pg/mL op dag twee, was dit geassocieerd met volledig herstel van orgaanfunctie op dag 7 en een verlaagde kans op overlijden (10% na 28 dagen). Echter, wanneer de bio-ADM spiegel op dag 2 hoger dan 70 pg/mL bleef was dit juist geassocieerd met meer orgaandisfunctie en een verhoogde kans op overlijden (38% na 28 dagen). Deze studie toont aan dat bio-ADM een prognostische waarde heeft die onafhankelijk van en van toegevoegde waarde is aan andere prognostische parameters. Verder laat dit onderzoek zien dat een tweede meting later in het opnametraject meerwaarde heeft voor het voorspellen van de prognose.

Deel III – Effecten van Adrecizumab in proefdieren

Eerder werd de effectiviteit van het adrenomedulline-antilichaam HAM1101 (de voorloper van Adrecizumab) reeds aangetoond in proefdiermodellen van systemische ontsteking en sepsis. Dit leidde tot ontwikkeling van het antilichaam Adrecizumab wat geoptimaliseerd was voor gebruik in mensen. In dit deel van het proefschrift worden de effecten van dit nieuwe geneesmiddel bij proefdieren beschreven.

In **hoofdstuk 9** werd de effectiviteit van Adrecizumab onderzocht in knaagdieren waarin een ontstekingsreactie werd opgewekt. In een eerste experiment werden 48 ratten behandeld met Adrecizumab of placebo, waarna endotoxine toegediend werd om een systemische ontstekingsreactie op te wekken. Vierentwintig uur later werden de ratten geofferd en werd het albuminegehalte in de nier en lever gemeten als maat voor vasculaire lekkage. Behandeling met 0.1 en 2.5 mg/kg Adrecizumab resulteerde in significant minder lekkage in de nier, maar niet in de lever, ten opzichte van placebo. Daarnaast resulteerde toediening van Adrecizumab in hogere adrenomedullineconcentraties in het bloed, hetgeen informatie verschaft over het werkingsmechanisme van Adrecizumab. In een ander experiment kregen 24 muizen Adrecizumab of placebo toegediend, gevolgd door een CLP (cecal ligation and puncture) operatie om sepsis te induceren. Tijdens deze ingreep wordt een stukje dikke darm afgebonden en doorgeprikt, waarbij feces de buikholte in lekt en een bacteriële sepsis ontstaat. In muizen die behandeld waren met Adrecizumab werd er verminderde expressie van het eiwit albumine (als teken van minder vaatlekkage) in de nier aangetoond. Verder kwam in Adrecizumab-behandelde muizen het eiwit VEGF (veroorzaakt meer capillair lek) verminderd tot expressie, terwijl het eiwit angiopoietin-1 (vermindert capillaire lekkage) juist verhoogd tot expressie kwam. Tot slot had Adrecizumab een gunstig effect op overleving in model van sepsis bij muizen. Een belangrijke kanttekening bij dit werk was dat Adrecizumab reeds toegediend werd voordat de proefdieren ziek gemaakt werden, hetgeen in de praktijk bij mensen uiteraard niet het geval kan zijn.

In **hoofdstuk 10** werden de effecten van Adrecizumab op hemodynamische parameters en hartfunctie onderzocht in ratten. Om sepsis te induceren ondergingen ratten de eerder genoemde CLP-operatie (of een 'nepoperatie' in een controlegroep). Dit werd 24 uur later gevolgd door intraveneuze toediening van Adrecizumab of placebo. Tevens waren er controlegroepen waarin ratten het bloeddrukverhogend geneesmiddel noradrenaline toegediend kregen, met of zonder Adrecizumab. Gedurende de eerste 3 uur na toediening van Adrecizumab of placebo werd de bloeddruk invasief gemeten en werd de hartfunctie ieder uur onderzocht middels echografie. Vervolgens werden de ratten geofferd en werden verschillende organen uitgenomen en nader geanalyseerd om ontstekingsmediatoren en oxidatieve stress te kwantificeren. Toediening van Adrecizumab resulteerde in een significante verhoging van de systolische bloeddruk en het hartminuutvolume. Het

tegelijktijdig gebruik van Adrecizumab en noradrenaline resulteerde niet in een verdere verbetering van hemodynamische parameters en hartfunctie. Vergelijkbaar met het onderzoek in het vorige hoofdstuk zorgde toediening van Adrecizumab voor een forse toename van bloedspiegels van adrenomedulline. Behandeling met Adrecizumab had geen effect op concentraties van diverse ontstekingsmediatoren in verschillende organen. Er werd daarentegen wel een significante vermindering gezien van oxidatieve stress in het hart van de ratten, hetgeen de gunstige effecten die gezien werden op de hartfunctie mogelijk kan verklaren.

In **hoofdstuk 11** werd het veiligheidsprofiel van Adrecizumab onderzocht bij ratten, honden en apen. Bij ratten en apen werd over een periode van twee weken 4 keer Adrecizumab toegediend. In het experiment met honden vonden eveneens herhaalde toedieningen plaats, maar dan over een langere periode van 28 dagen. De veiligheidsanalyse bestond onder andere uit het registreren van eventuele bijwerkingen, analyse van bloed en urine, en (histo)pathologisch onderzoek. In de hondenstudie lag de nadruk op de evaluatie van hemodynamische parameters. Uit deze experimenten kwamen geen veiligheids-issues naar voren die gerelateerd konden worden aan de toediening van Adrecizumab. Alle toegepaste doseringen (tot 400 mg/kg/dag in ratten, tot 50 mg/kg in honden en tot 100 mg/kg/dag in apen) werden goed getolereerd. Farmacokinetische analyse liet onder andere zien dat Adrecizumabconcentraties proportioneel stegen in relatie tot de toegediende dosering en dat het middel een lange halfwaardetijd heeft, zoals vaker gezien wordt bij antilichamen. Net als in de voorgaande hoofdstukken steeg het adrenomedullinegehalte in het bloed sterk na de toediening van Adrecizumab, hetgeen niet gepaard ging met een lagere bloeddruk in honden en apen.

Deel IV – Effecten van Adrecizumab bij de mens

Op basis van de voorgaande resultaten in dieren werd besloten om nader onderzoek te verrichten bij de mens. In **hoofdstuk 12** werd Adrecizumab voor het eerst aan mensen toegediend. In een zogeheten *first-in-human* studie hebben we de veiligheid, farmacokinetiek en andere effecten van Adrecizumab onderzocht bij gezonde vrijwilligers. In een tweede studie werden deze parameters onderzocht in het experimentele endotoxinemiemodel, waarbij endotoxine toegediend werd aan gezonde vrijwilligers om een systemische ontstekingsreactie op te wekken. In totaal hebben 48 gezonde mannelijke

vrijwilligers deelgenomen aan deze 2 dubbelblind gerandomiseerde placebogecontroleerde studies. In beide studies kregen de proefpersonen 0.5, of 2, of 8 mg/kg Adrecizumab, of placebo (n=6 per groep) toegediend. Omdat de halfwaardetijd van Adrecizumab in de dierstudies erg lang was, werden alle proefpersonen gedurende een periode van 90 dagen opgevolgd. Beide studies lieten een uitstekend veiligheidsprofiel zien van Adrecizumab. Analyse van de farmacokinetiek toonde aan dat Adrecizumabconcentraties in het bloed op een proportionele manier stegen in relatie tot de toegediende dosering, net zoals in de dierstudies. Daarnaast werd een klein verdelingsvolume, een langzame klaring en een halfwaardetijd van ongeveer 14 dagen waargenomen, hetgeen zeer vergelijkbaar is met andere antilichaammedicijnen. De door endotoxine opgewekte ontstekingsreactie in de 2e studie had geen relevante invloed op deze farmacokinetische parameters. Net zoals in de dierstudies (hoofdstuk 9 t/m 11), leidde toediening van Adrecizumab tot een sterke stijging van adrenomedullinespiegels in het bloed. Adrecizumab had geen effecten op de endotoxinemie-geïnduceerde daling van bloeddruk, temperatuurstijging of klaring van ontstekingsmediatoren. Dit kwam niet als een verrassing, omdat de opzet van de studie was gericht op veiligheid en farmacokinetiek, waarbij Adrecizumab pas 1 uur na start van LPS-infusie toegediend werd, op een moment dat de ontstekingsreactie al in gang gezet was. Desalniettemin werd er een significant lagere symptoomscore gezien in vrijwilligers die behandeld werden met 8 mg/kg Adrecizumab, en werd er een vergelijkbare trend gezien bij de lagere dosisgroepen.

De resultaten bij proefdieren en vrijwilligers waren dusdanig veelbelovend dat besloten werd de veiligheid en effectiviteit van Adrecizumab bij patiënten met septische shock te onderzoeken. In **hoofdstuk 13** wordt het studieprotocol van de AdrenOSS-2 studie beschreven, een internationale dubbelblind gerandomiseerde, placebogecontroleerde, multicenter, proof-of-concept en dose-finding fase II *first-in-patient* studie. Het primaire eindpunt van de studie is de veiligheid van Adrecizumab. Om dit te onderzoeken worden patiënten tot 90 dagen na toediening opgevolgd. De studie heeft een aantal interessante kenmerken. Zo wordt het bio-ADM gehalte in het bloed van patiënten gebruikt als een inclusiecriterium: alleen patiënten met een verhoogd gehalte (> 70 pg/mL) mogen deelnemen aan de studie. Omdat dit geassocieerd is met een ongunstiger ziektebeloop, een lagere bloeddruk, meer vaatlekkage en een hogere kans op overlijden, kan dit van nut zijn om patiënten te identificeren die het meeste baat zouden kunnen ondervinden

van behandeling met Adrecizumab. Het is uniek voor een sepsistrial dat een biomarker als inclusiecriterium wordt toegepast en een voorbeeld van personalized medicine. Daarnaast wordt gebruik gemaakt van een nieuw eindpunt: de sepsis support index. Dit eindpunt combineert sterfte en een orgaandisfunctiescore over een periode van 14 dagen. Er wordt verwacht dat dit eindpunt gevoeliger (en daardoor meer geschikt) is om een mogelijk effect van Adrecizumab aan te tonen, vergeleken met enkel sterftecijfers. Er zullen in totaal 300 patiënten geïncludeerd worden in 30 deelnemende centra in Europa, waarbij 2 doseringen Adrecizumab onderzocht worden (2 en 4 mg/kg). De studie is in december 2017 van start gegaan en de resultaten worden in eind 2019 verwacht.

Conclusie

Adrenomedulline speelt een belangrijke rol in het ziektebeeld sepsis door beïnvloeding van de bloeddruk en lekkage van bloedvaten. Bloedspiegels van bioactief adrenomedulline hebben een voorspellende waarde voor het ziektebeloop van IC patiënten en het is van meerwaarde om deze spiegel later in het opnametraject opnieuw te bepalen. Het experimentele geneesmiddel Adrecizumab lijkt een veelbelovende behandeling voor patiënten met septische shock. In proefdieren en gezonde vrijwilligers laat het een uitstekend veiligheidsprofiel zien. Daarnaast is er een plausibele hypothese voor het werkingsmechanisme van Adrecizumab, wat nog wel nader onderzoek vereist in de toekomst. Adrecizumab zou daarnaast ook gunstige effecten kunnen hebben bij ziektebeelden anders dan sepsis, waarbij eveneens disfunctie van hart en bloedvaten optreedt, zoals hartfalen, dengue of andere capillair leksyndromen. Momenteel wordt onderzocht of Adrecizumab daadwerkelijk effectief is bij patiënten met septische shock in de AdrenOSS-2 studie, waarvan de resultaten binnenkort volgen.

Appendices

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Christopher Geven werd geboren op 29 september 1990 te Asten. In 2008 behaalde hij zijn VWO-diploma aan het St.-Willibrord Gymnasium te Deurne. Aansluitend begon hij met zijn studie Geneeskunde aan de Radboud Universiteit Nijmegen waar hij in 2014 zijn artsexamen behaalde. Vervolgens is hij een jaar werkzaam geweest als arts-assistent op de afdeling Intensive Care van het Radboudumc in Nijmegen. In 2015 is hij onder begeleiding van dr. Matthijs Kox en prof. dr. Peter Pickkers gestart met zijn promotie onderzoek naar het nieuwe experimentele geneesmiddel Adrecizumab, hetgeen resulteerde in de totstandkoming van dit proefschrift. Tijdens zijn promotietraject heeft hij tevens een korte periode onderzoek gedaan in het Hôpital Lariboisière in Parijs onder begeleiding van prof. dr. Alexandre Mebazaa. De bevindingen voortkomend uit dit medisch wetenschappelijk onderzoek heeft hij gepresenteerd op diverse congressen in binnen- en buitenland.

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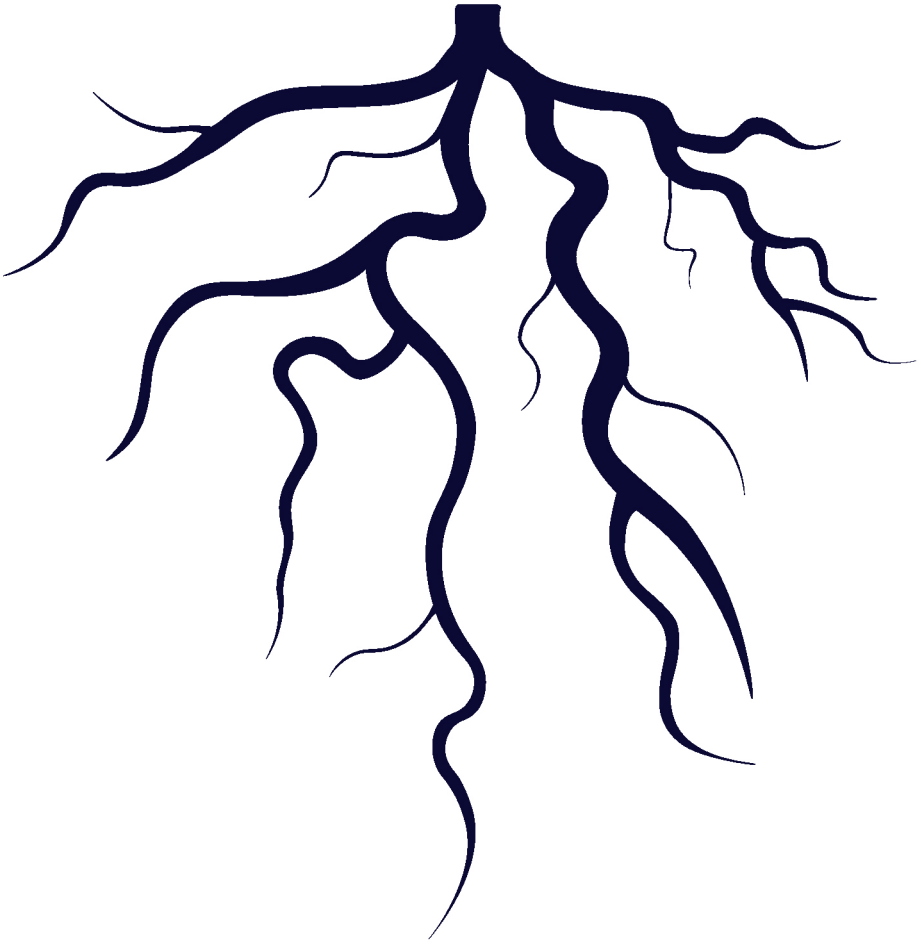
PHD PORTFOLIO

Name PhD candidate: C. Geven *Department: Intensive Care Medicine* *Graduate School: Radboud Institute for Molecular Life Sciences*	*PhD period: 15-01-2016 – 15-03-2019* *Promotor: Prof. dr. P. Pickkers* *Co-promotor: Dr. M. Kox*	
	Year(s)	ECTS
TRAINING ACTIVITIES		
a) Courses & Workshops		
- *RIMLS introduction*	2016	1.0
- *BROK*	2016	1.5
- *IC research journal club*	2016, 2017	2.0
- *Statistics course*	2016	0.2
- *How to sell your science for PhD students*	2016	0.2
- *Scientific integrity course*	2018	1.0
b) Seminars & lectures		
- *Nederlandse Vereniging Voor Intensive Care (NVIC) congres (poster, oral)*	2017, 2018	0.5
- *RIMLS PhD retreat (poster)*	2017	0.25
- *European Society of Intensive Care Medicine (ESICM) congress (2 oral presentations)*	2017	1.0
- *Science Day RCI (oral presentation)*	2017	0.25
- *Calcitonin gene-related peptide (CGRP) conference (oral)*	2018	0.5
c) Symposia & congresses		
- *Radboud Spring Frontiers Host-directed therapy in infectious diseases (Nijmegen)*	2016	0.5
- *PhD Retreat RIMLS (Veldhoven)*	2016, 2017	1.0
- *Nederlandse Vereniging Voor Intensive Care (NVIC) congres (Amsterdam)*	2016-2018	1.5
- *European Society of Intensive Care Medicine (ESICM) congress (Milaan, Wenen)*	2016, 2017	1.5
- *Afscheidssymposium Eric van Leeuwen (Nijmegen)*	2017	0.2
- *Science day Radboudumc-AMC (Leuteren)*	2017	0.25
- *Science day RCI (Groesbeek)*	2017	0.25
- *CGRP conference (Santa Fe, USA)*	2018	0.75
- *ISICM (Brussels)*	2018	0.25
d) Other		
- *Co-organizing IC research weekend*	2016	
- *Research meetings Intensive Care, Radboudumc*	2016-2018	
TEACHING ACTIVITIES		
e) Supervision of internships / other		
- *Medical students (3x)*	2016-2018	3.5
- *Reviewing of scientific publications (6x)*	2016-2018	0.6
TOTAL		**18.7**



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